REMARKS

In the Office Action dated April 27, 2007, claims 45-132 are pending. The Examiner has made the Restriction Requirement final. Consequently, claims 66-67, 72-86 and 92-132 are withdrawn from further consideration, and claims 45-63, 65, 68-71 and 87-91 are under consideration and are rejected.

This Response addresses each of the Examiner's rejections and objections. Applicant therefore respectfully submits that the present application is in condition for allowance. Favorable consideration of all pending claims is therefore respectfully requested.

Claims 68, 69, 90 and 91 are objected to under 37 C.F.R. §1.75(c) as improper because these claims depend on non-elected claims. Therefore, the Examiner has not considered these claims on the merits.

Applicant has amended the claims such that 68, 69, 90 and 91 depend on elected claims. Withdrawal of the objection to these claims and examination thereof are respectfully requested.

Claims 45-47, 56-58, 62-64, 71 and 87-88 are rejected under 35 U.S.C. §102(b) as anticipated by Wobus et al. (*Roux's Arch Dev Biol* 204: 36-45, 1994; hereinafter "Wobus (1994)"). The Examiner contends that Wobus (1994) teaches a method for controlling the differentiation of the D3 mouse embryonic stem cells into cardiomyocytes depending on the concentration and treatment time of retinoic acid (RA).

Further, Claims 45-46, 70 and 87 are rejected under 35 U.S.C. §102(b) as anticipated by Wobus et al. (*J Mol Cell Cardiol* 29: 1525-1539, 1997; hereinafter "Wobus (1997)"). The Examiner contends that Wobus (1997) teaches culturing the mouse embryonic

stem cell line D3 in undifferentiated state on feeder layer of primary mouse embryonic fibroblasts and their differentiation into cardiomyocytes after RA treatment.

Applicant respectfully submits that the newly amended claims are directed to inducing differentiation of human embryonic stem (hES) cells by culturing the hES cells in the presence of an embryonic cell and/or extracellular medium of an embryonic cell. Both Wobus (1994) and Wobus (1997) relate to the differentiation of mouse stem cells. Moreover, as disclosed in both references, the differentiation is induced by the presence of retinoic acid (RA), which is distinguished from a co-culture of the hES cells in the presence of embryonic cells and/or extracellular medium thereof. Accordingly, neither reference teaches each and every element of the claimed invention. Withdrawal of the anticipation rejections based on Wobus (1994) and Wobus (1997), respectively, is respectfully requested.

Claims 45-51, 53-54 and 60-63, are rejected under 35 U.S.C. §102(b) as anticipated by Mummery et al. (*Differentiation* 46: 51-60, 1991) (hereinafter "Mummery (1991)").

According to the Examiner, Mummery (1991) teaches that when P19 embryonal carcinoma (EC) cells were co-cultured with cells from one of several established visceral-endoderm-like cell lines, the EC cells were rapidly induced to aggregate and differentiate into cell types including mesoderm-derived cardiac and skeletal muscle.

Applicant respectfully submits that Mummery (1991) relates to the co-culture of embryonic cells with P19 mouse embryonal carcinoma (EC) cells. Because the present claims are directed to culturing human ES cells, the reference does not teach each and every element of the claimed invention. Therefore, the anticipation rejection based on Mummery (1991) is overcome. Withdrawal of the rejection based on Mummery (1991) is respectfully requested.

Claims 45-46 and 59 are rejected under 35 U.S.C. §102(b) as anticipated by Gearhart et al. (WO 98/43679) (hereinafter "Gearhart").

According to the Examiner, Gearhart teaches collection of human primordial germ cells (PGC) and derivation of embryonic germ cells derived from human aborted fetal material. Gearhart also allegedly teaches that the human PGC and STO mouse fibroblasts were cultured and human EG cells were obtained; and cardiomyocytes were subsequently generated from human EG cells.

Applicant respectfully submits that the methods as presently claimed, are distinguished from the method disclosed by Gearhart in several aspects. In the first instance, Gearhart teaches that cardiomyocytes are derived from human embryonic germ (EG) cells. In contrast, the newly amended claims are directed to culturing and differentiating human ES cells, which are distinct from human EG cells. EG cells are derived from Primordial Germ Cells (PGC), which in turn may be derived from gonadal ridges of the embryo. ES cells, on the other hand, are derived from the inner cell mass of an embryo.

In addition to the differences in the cells being cultured, the ways of how the cells are being cultured and induced to differentiate are also different between the claimed method and that disclosed by Gearhart. As presently claimed, the hES cells are cultured in the presence of an embryonic cell and/or extracellular medium of an embryonic cell. Such co-culture induces differentiation of the hES cells. On the other hand, according to Gearhart (see, for example, page 28 of Gearhart), the cardiomyocytes are produced from embryoid bodies that were developed from undifferentiated EG cells grown on primary embryonic fibroblast layers. However, the primary embryonic fibroblast layers are used only for the purpose of maintaining the EG cells in an undifferentiated state. Differentiation of the EG cells does not happen until

the cells are removed from the fibroblast layers and allowed to differentiate to develop an embryoid body. Hence, in this citation, it is clear that no differentiating signals are provided by the primary embryonic fibroblast layers.

In view of the foregoing, it is respectfully submitted that Gearhart does not teach each and every element of the present invention. Thus, the rejection under 35 U.S.C. §102(b) based on Gearhart is overcome, and withdrawal thereof is respectfully requested.

Claim 90 is rejected under 35 U.S.C. §102(b) as anticipated by Rohwedel et al. (*Dev Biol.* 164(1): 87-101, 1994; hereinafter "Rohwedel"). The Examiner contends that Rohwedel teaches a skeletal muscle cell derived by culturing embryonic stem cells after applying external signals.

Applicant respectfully submits that Rohwedel utilizes mouse embryonic stem cells to prepare skeletal muscle cells. The skeletal muscle cell of claim 90, as amended, is a human skeletal muscle cell prepared from a human embryonic stem cell. Accordingly, Rohwedel does not anticipate the subject matter of claim 90. Withdrawal of the §102(b) rejection based on Rohwedel is respectfully requested.

Claim 91 is rejected under 35 U.S.C. §102(b) as anticipated by Mummery (1991).

Applicant respectfully submits that Mummery (1991) utilizes P19 EC cells, which are mouse embryonal carcinoma cells. In contrast, any vascular endothelial cells prepared by the method according to claim 45 would in fact be human cells, and not mouse cells described in Mummery (1991). Hence, Mummery (1991) does not anticipate claim 91. Withdrawal of the §102(b) rejection based on Mummery (1991) is respectfully requested.

Claims 55, 65 and 89 are rejected under 35 U.S.C. §103(a) as unpatentable over Mummery (1991) in view of Mummery et al. (*Biochem Biophys Res Commun* 191(1): 188-195, 1993).

Mummery (1991) allegedly teaches the use of a P19 (mouse) embryonal carcinoma (EC) cells in a co-culture with visceral-endoderm like cells. The Examiner admits that Mummery (1991) differs from the claimed invention by not teaching differentiation of a human stem cell into a cardiomyocyte.

However, the Examiner contends that at the time the claimed invention was made, Mummery (1993) teaches that the differentiation of blastocyst stage mouse embryonic stem cells in culture is regulated by the fibroblast growth factor and its receptor. Mummery (1993) also allegedly teaches that FGF-receptor isoforms are also expressed on the human embryonal carcinoma, which are undifferentiated stem cells of human teratocarcinomas, similar to the blastocyst stage mouse embryonic stem cells. Mummery also allegedly teaches that, in contrast to murine cell lines, human EC cells express several other classes of growth factor receptors. Mummery (1993) suggests that since all these factors are expressed by the undifferentiated cells in both human and mouse embryonic stem cells, these cells provide a useful model to study changes in the FGF-mediated signal transduction pathways during differentiation. As such, the Examiner contends that Mummery (1993) provides sufficient motivation for one of ordinary skill in the art to apply the mouse culture system of Mummery (1991) for studying signal transduction pathways in the human EC cells. The Examiner is also of the view that those skilled in the art would have had a reasonable expectation of success to modify the mouse system, disclosed by Mummery (1991), to a human system.

Applicant respectfully disagrees with the Examiner's rejection and submits that the subject matter, as presently claimed, is not obvious because the present invention is directed to differentiation of human ES cells, which are distinguished from the mouse EC cells utilized in Mummery (1991) as well as the human EC cells disclosed in Mummery (1993). The Examiner has not provided a reason or motivation for those skilled in the art to combine the teaching of mouse EC cells with that of human EC cells to arrive at instant human ES cells, and to do so with a reasonable expectation of success.

In the first instance, human ES cells are different from stems cells of other species, and one cannot always extrapolate the results from one species to another species. For instance, mouse ES cells are quite different from human ES cells, and require different culturing conditions. Mouse ES cells require LIF whereas human ES cells can be cultured in the absence of LIF. Therefore, the conditions that may be relevant to ES cells from one species (e.g., mouse) would not necessarily apply to ES cells from another species (e.g., human).

Furthermore, Mummery (1991) is directed to culturing mouse embryonic carcinoma (EC) cells, and the portion of Mummery (1993) relied on by the Examiner is directed to human EC cells. EC cells are distinct from embryonic stem (ES) cells employed in the present invention. EC cells are derived from malignant teratocarcinomas. They are generally karyotypically abnormal, including aneuploidy. In other words, EC cells do not carry a normal complement of chromosomes (*Appendix C, C-8, Stem Cells: Scientific progress and future research* directions –NIH publication June 2001, available online at http:\\stemcells.nih.gov/info/scireport/appendixC.asp). When differentiated, EC cells show an inability to differentiate into well-recognized cell types. Epigenetic changes allow these cells to adapt to efficient tumor growth. Even when transplanted, EC cells retain the ability to form

teratocarcinomas. EC cells are generally known as the malignant counterpart of ES cells or cells of the inner cell mass (ICM), and generally do not differentiate significantly to a diversity of cells (Andrews (2002) *Phil. Trans R. Soc. Lond.* B, 357, 405 - 417) **(Exhibit 1)**.

Conversely, ES cells are considered "genetically normal" as are the differentiated cell types that arise from them. ES cells are derived from the ICM of blastocyst stage embryos. Since ES cells are karyotypically normal, when grown in immunodeficient mice, they form well-defined teratomas with well-organized tissues representing all the three germ layers (as shown in Thomson et al., *Science* 282:1145-1147, 1998). In addition, when ES cells are injected into a mouse blastocyst and the blastocyst is returned to the uterus, chimeric mice are formed in which most, if not all tissues (including the germ cells), contain progeny of the donor ES cells. In contrast, EC cells rarely contribute to many cells types in the chimera and additionally rarely if ever form the germ tissue as do the hES cells. The ability of hES cells to contribute so widely to different cell types either in teratomas or chimeras is a defining characteristic of hES cells that demonstrates their true pluripotent nature (See, e.g., Andrews (2002), **Exhibit 1**, page 411, right column, second paragraph).

Other differences between the EC and ES cells can be seen in surface antigens detected by antibodies GD3 and GD2. Surface antigens such as A2B5 and ME311 are induced at relatively low levels in ES cells, whereas in EC cells these can be induced significantly.

Accordingly, the physical characteristics and the differentiation profiles of EC cells and ES cells are quite different. The uncontrolled nature of EC cells, particularly their underlying karyotypically abnormal nature, makes it especially difficult to predict the behavior of these cells and to compare against other karyotypically normal cell types such as ES cells.

In this connection, it has been reported by Draper et al. (2002) that EC cells generally are not capable of mesodermal differentiation (Draper et al., *J. Anat.* 200: 249-258, 2002, particularly page 256, line 12 from the bottom) (**Exhibit 2**). Draper et al. (2002) refer to the EC cell line NTERA-2, which was shown to be incapable of mesodermal differentiation by Gokhale et al. (*Cell Growth Diff* 11: 157 -162, 2000) (**Exhibit 3**). In contrast, as shown in the present invention, hES cells are capable of being differentiated in a controlled and reproducible manner into cells of the mesodermal lineage, i.e. the cardiomyocytes of the present invention.

Therefore, in light of the inability of certain EC cells to differentiate into cells of the mesodermal lineage, as reported in the art especially Draper (2002) and Gokhale et al. (2000), the results achieved by the present invention, namely, differentiation of human ES cells into cells of the mesodermal lineage, are quite surprising. Apparently, hES cells, although still regarded as a tissue culture artefact, are far more representative of a normal pluripotent cell type that EC cells. It is clear that the results achieved with EC cells cannot be readily extrapolated to hES cells.

The Examiner has also relied on Mummery (1993) and has reasoned that because human EC cells express FGF receptors, these cells would behave in the same manner as human ES cells which also express FGF receptors.

Applicant respectfully submits that it is improper for the Examiner to conclude that human EC cells and human ES cells would behave the same way simply because both cell types express FGF receptors. Applicant respectfully submits that the process of differentiation is a very complex one, and is unlikely to be regulated by one growth factor alone.

Contrary to the Examiners assertions, Applicant respectfully submits that those skilled in the art would not have had any reasonable expectation of success in modifying the

mouse EC system to the human ES system because, as submitted above, human ES cells are different from mouse ES cells, ES cells are distinct from EC cells, and the results of EC cells are not predictive of ES cells. Therefore, Applicant respectfully submits that the presently claimed invention, as a whole, is not obvious over the cited references. Withdrawal of the rejection under 35 U.S.C. §103(a) based on Mummery (1991) in view of Mummery (1993) is respectfully requested.

Claims 45-46 are rejected on the ground of nonstatutory obviousness-type double patenting as allegedly unpatentable over claim 1 of Serial No. 11/644,790.

Applicant respectfully submits that the claims, as presently amended, are patentably distinct over claim 1 of Serial No. 11/644,790. As such, withdrawal of the double patenting rejection is respectfully requested.

Applicant acknowledges the Examiner's determination that claims 52, 68 and 69 are free of the prior art.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,

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From teratocarcinomas to embryonic stem cells

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The recent derivation of human embryonic stem (ES) cell lines, together with results suggesting an unexpected degree of plasticity in later, seemingly more restricted, stem cells (so-called adult stem cells), have combined to focus attention on new opportunities for regenerative medicine, as well as for understanding basic aspects of embryonic development and diseases such as cancer. Many of the ideas that are now discussed have a long history and much has been underpinned by the earlier studies of teratocarcinomas, and their embryonal carcinoma (EC) stem cells, which present a malignant surrogate for the normal stem cells of the early embryo. Nevertheless, although the potential of EC and ES cells to differentiate into a wide range of tissues is now well attested, little is understood of the key regulatory mechanisms that control their differentiation. Apart from the intrinsic biological interest in elucidating these mechanisms, a clear understanding of the molecular process involved will be essential if the clinical potential of these cells is to be realized. The recent observations of stem-cell plasticity suggest that perhaps our current concepts about the operation of cell regulatory pathways are inadequate, and that new approaches for analysing complex regulatory networks will be essential.

Keywords: embryonic stem cells; teratocarcinoma; embryonal carcinoma; human; differentiation; plasticity

1. INTRODUCTION

The present flurry of interest in stem cells and their potential for 'regenerative' medicine has arisen from the confluence of several streams of biological thought and investigation. The first is the notion of stem cells themselves, which evolved among biologists considering the regeneration of tissues that are necessarily replaced throughout adult life, most obviously the blood, the lining of the gut and the skin. It was proposed that the replacement of these complex tissues, comprising many different cell types, depends upon the existence of small populations of undifferentiated cells, 'stem cells', that not only proliferate to replace themselves indefinitely ('self-renewal'), but also differentiate into daughter cells with a limited proliferative capacity. These so-called 'transit amplifying' cells are committed to differentiate further to a restricted range of terminal cell types responsible for the principal functions of such tissues (Lajtha 1979; Potten & Lajtha 1982). Such terminally differentiated cells typically lose their capacity for further proliferation.

This type of tissue architecture may very well have a selective advantage in minimizing the susceptibility of an organism to cancer, which is almost certainly the result of the accumulation of mutations that affect both cell proliferation and differentiation. It was pointed out by Pierce (1974) that many cancers seem to be caricatures of their tissue of origin, with a malignant stem cell similar to the stem cell of the healthy tissue, as well as a variety of cells at progressive stages of differentiation. He proposed that malignancy is a consequence of a failure to regulate the balance between proliferation and differentiation, resulting in the accumulation of cells at earlier stages of the differentiation pathways. Where the issue has been analysed in detail, notably in the haematological malignancies, possibly only few cells within a tumour mass, the so-called 'clonogenic cells', are capable of transferring a malignancy to a new host (Greaves 1982). Cairns (1975) pointed out that it is mutations occurring in the stem-cell population that are most likely to become 'fixed' in a tissue and cause long-term damage because mutations in transit amplifying and terminally differentiated cells would normally be lost as the tissue turned over. He suggested that there would then be a selective advantage for an organism to maintain only small stem populations to minimize the target for mutagenesis, and hence carcinogenesis.

Many tissues that generally appear quiescent also turn over slowly (e.g. bone), or may proliferate or regenerate in response to physiological cues (mammary tissue in pregnancy) or injury (liver). Presumably these, too, retain stem cells, or cells that are capable of conversion into stem cells. But it has now become evident that tissues not previously thought capable of regeneration, notably the nervous system, also harbour stem-cell populations (Temple 2001). However, the results that have caused general surprise come from a series of experiments, by different groups working on different organ systems, suggesting that stem cells from one tissue, hitherto thought committed to the lineages of that tissue, can give rise to distinct cell types of embryologically unrelated tissues under some circumstances. Thus, evidence has been presented for neural stem cells giving rise to blood cells (Bjornson *et al.* 1999) and to muscle (Galli *et al.* 2000), haematopoietic stem cells giving rise to neurons (Brazelton *et al.* 2000; Mezey *et al.* 2000), muscle (Goodell *et al.* 2001) and liver (Alison

et al. 2000; Peterson *et al.* 1999; Lagasse *et al.* 2000), muscle stem cells giving rise to blood cells (Jackson *et al.* 1999; Seale *et al.* 2001), and mesenchymal stem cells from bone giving rise to a wide variety of tissues (Pittenger *et al.* 1999). Perhaps the most striking observation was that neural stem cells transferred to a blastocyst could contribute to tissues belonging to all three germ layers (Clarke *et al.* 2000).

Many questions still remain about these observations of apparent stem-cell plasticity; only in a few cases have the experiments been conducted with clonal, well-characterized stem cells—a necessity if one is to conclude that a particular stem cell is able to give rise to several distinct cell types. In any case, if we take the results at face value, are we observing a normal physiological function of stem cells, or are we seeing the consequences of rare switches in their differentiative capacity or potency? In the latter case, is this because of the exposure of the cells to environments that they do not normally see—an issue of the difference between what cells 'can do'—'prospective potency'—and what they normally do when undisturbed—'prospective fate' (Weiss 1939). Or are the changes in their patterns of differentiation due to rare events involving intrinsic changes to the patterns of gene activity that regulate the determined state of a cell? Regardless of the answers to these questions, the observations have nevertheless raised the prospect of clinically using stem cells from adult tissues in cell replacement therapies designed to repair or replace tissues damaged by accident or disease.

The second stream of work contributing to the current debate is one that began with a long-standing fascination with a peculiar type of tumour, the teratomas, and has culminated in the derivation of stable, pluripotent human embryonic stem (ES) cell lines in culture. Teratomas present a haphazard array of cell differentiation that appears to recapitulate many of the events that occur during early embryonic development but in a disorganized manner (Damjanov 1993; Dixon & Moore 1952; Mostofi & Price 1973). They have intrigued clinicians and biologists for many hundreds of years; excellent summaries of the history of this interest are provided by Damjanov & Solter (1974) and Wheeler (1983). The experimental study of teratomas began with the discovery by Stevens & Little (1954) that they occur spontaneously in the testes of about 1% of male mice of the 129 strain, or that they can be induced by transplanting the genital ridges of embryos from strain 129 mice, and a few other strains, to the testes of adult mice (Stevens 1964, 1970*a*,*b*). Some of these tumours were evidently malignant and could be re-transplanted to successive hosts. Such 'teratocarcinomas', as the re-transplantable, malignant tumours were known, contained a relatively undifferentiated cell type known as an 'embryonal carcinoma' (EC) cell, long suspected as the stem cell of the tumour. This stem-cell character of EC cells was confirmed by Kleinsmith & Pierce (1964), who showed that single EC cells transferred to a new host could reform a complex teratocarcinoma that could again be re-transplanted to another host. A long series of experiments through the 1960s and 1970s, seeking to characterize these EC cells and their relationship to embryonic cells, culminated with the isolation, in 1981, of pluripotent cells from very early mouse embryos (Evans & Kaufman 1981; Martin 1981) and eventually, in 1998, from human embryos (Thomson *et al.* 1998). These 'normal' embryo-derived cells became known as ES cells. In fact, the term 'stem cell' was rarely used previously by embryologists (e.g. Wilson 1896) and it then generally referred to precursor cells within the developing embryo. Such cells usually exist only transiently, and their self-renewal is normally limited, unlike 'stem cells' in the adult. Although ES cell lines in culture do meet the criteria used in the context of 'adult stem cells' of indefinite self-renewal together with a capacity for differentiation, the embryonic cells of the late inner cell mass (ICM), to which they correspond, generally disappear from the developing embryo by the time of gastrulation. However, it is intriguing to speculate that a number of childhood cancers may arise because of the abnormal persistence of 'stem cells' present during embryogenesis.

The third stream of relevant research encompasses the work of those who sought first to determine whether terminal differentiation of cells during embryogenesis results from the loss of genes, or whether altered regulation without a significant change in the complement of genes present in a cell could be responsible. Following earlier studies of Briggs & King (1952), Gurdon (1962) showed that, in *Xenopus laevis*, nuclear transfer from differentiated somatic cells to enucleated oocytes supported embryonic development, indicating that the nuclei of differentiated cells in amphibians contain a full genetic complement. These experiments incidentally demonstrated an approach for producing 'clones' of genetically identical individuals. For many years it seemed that this might not be possible in the case in mammals (McGrath & Solter 1984), and definitive evidence that mammalian 'cloning' by nuclear transfer from somatic cells is possible was not acquired until experiments with sheep embryos led to the birth of 'Dolly', born following the transfer of a nucleus from a mammary cell of an adult sheep to an enucleated oocyte (Campbell *et al.* 1996; ilmut *et al.* 1997). That observation was quickly followed by finding that 'cloning' is also possible in mice (Wakayama *et al.* 1998), cows (Cibelli *et al.* 1998) and pigs (Onishi *et al.* 2000). It is a short step to imagine that this would also be possible in humans, if anyone chose to take this step.

The idea of using such nuclear transfer techniques for so-called 'reproductive cloning' in humans is an anathema to most scientists, not least because the process is both inefficient and subject to serious errors so that most animals developing by this route have proved to be defective. However, it rapidly occurred to several people that embryo cloning by this route, coupled with the ability to derive ES cells from very early embryos at the blastocyst stage, could allow the development of almost any differentiated cell type that would be genetically identical to a prospective patient, who could then receive such cells in tissue replacement therapies (essentially autografts), the so-called 'therapeutic cloning' approach. As a proof of concept, Munsie *et al.* (2000) have isolated murine ES cells from blastocysts derived by somatic nuclear transplantation. The derivation of human embryos following somatic nuclear transfer to an enucleated oocyte has also been reported, though subsequent development arrested prior to blastocyst formation at the six-cell stage (Cibelli *et al.* 2001). So far, no one has described the derivation

of human ES cells from such embryos. Whether it will ever prove a practicable proposition to derive cells tailored for specific patients, instead of finding other ways for defeating the immune system to permit allografts from established, non-autologous ES lines, is a moot point.

Thus, the current excitement about the potential of stem-cell biology for regenerative medicine arises in part from the identification and culture of various types of stem cells, whether from embryos or adults, and in part from results indicating that adult stem-cell plasticity or embryo cloning by somatic cell nuclear transplantation may be realistic approaches for providing autologous tissues for grafting to evade problems of immunological incompatibility. Nevertheless, it is arguable that, despite considerable progress in the biology of stem cells from various adult tissues, ES cells from the early embryo are perhaps the best understood. Clear identification of the various adult stem cells has often been elusive; some, notably haematopoietic stem cells, cannot be easily cultured and expanded *in vitro*, while others require continual re-isolation as indefinite culture *in vitro* does not seem possible. By contrast, ES cells from mice and humans have been cloned (Amit *et al.* 2000), in the sense that it has been shown that single identifiable cells can give rise to a variety of distinct cell types by differentiation, they can be cultured and expanded *in vitro*, apparently indefinitely, without loss of potency, and they could, in principle, be used to generate all tissues of the body. These features mean that it should be possible establish an inexhaustible supply of well-characterized cells for clinical use—an important issue for ensuring safe protocols. It is also important to recognize that ES cells, and their malignant EC cell counterparts, provide invaluable tools for analysing cell differentiation throughout embryogenesis, which also has implications for understanding diseases such as cancer. In turn, ES cells may also provide keys for understanding the biology of 'adult stem cells'.

2. THE BIOLOGY OF TERATOCARCINOMAS

Teratomas are generally benign tumours that occur most commonly in the ovary, where they are also known as benign ovarian cysts (figure 1*a*). These arise from oocytes that have undergone parthenogenetic activation, begun development and then become disorganized to form a mass of embryonic tissue (Stevens & Varnum 1974). Teratomas are also found, though more rarely, in other sites, including the base of the spine in newborn infants.

Similar tumours occur in the testis, but in this case they are generally highly malignant and consequently known as teratocarcinomas (Dixon & Moore 1952; Mostofi & Price 1973). These form a subgroup of the germ-cell tumours (GCTs) that account for almost all testicular cancers. Testicular GCTs, which appear to arise from abnormal gonocytes within the seminiferous tubules (figure 1*b*) (Skakkebaek 1972) are rare, but have a peak incidence in young post-pubertal men making them the most common malignancy in this age group (Møller 1993). Furthermore, their incidence has increased dramatically over the past 50 years. The uncommonly young age of GCT patients contributes to their medical significance but, fortunately, GCT are amongst the most treatable cancers since the advent of *cis*-platinum-based therapy in the 1970s (Einhorn 1987; Stoter 1987).

GCTs are typically divided into seminomas and non-seminomas (Damjanov 1990, 1993). Seminomas consist of cells that resemble primordial germ cells; they do not occur in mice. In contrast, non-seminomas are histologically heterogeneous and frequently contain somatic tissues such as nerve, bone, muscle, etc.; sometimes they contain structures, embryoid bodies, in which these cells are organized to resemble closely an early embryo (figure 1*c*,*d*). These tumours also contain histologically undifferentiated elements composed of EC cells, the key malignant pluripotent stem cell of these tumours. The term teratocarcinoma is generally used for tumours containing both EC and teratoma components.

In 1954, Stevens & Little reported that males of the 129 strain develop spontaneous testicular teratomas and teratocarcinomas that can be observed as incipient tumours, forming structures described as embryoid bodies within the seminiferous tubules of the developing gonad, as early as 13 days of embryonic development (Stevens 1964). Stevens also showed that these tumours can be induced experimentally in strain 129 mice and a limited range of other strains by explanting genital ridges of foetuses, between 11 and 13.5 days of development, to ectopic sites (Stevens & Hummel 1957; Stevens 1967*a*, 1970*a*). These results suggested that the origins of teratomas were from primordial germ cells as, in the mouse embryo, these migrate into the genital ridge at 11 days (Bendel-Stenzel *et al.* 1998). The upper limit of 13 days implies some further changes in the germ cells, which could be associated with their entering mitotic arrest soon after their arrival in the genital ridge. Confirmation of the germ-cell origin of the spontaneous and experimental testicular teratomas came from studies of mice homozygous for the *Steel* (*Sl*) mutation (Stevens 1967*b*). Viable *Sl*/*Sl* homozygotes are infertile as the primordial germ cells do not survive migration. Stevens found that the genital ridges from homozygous *Sl*/*Sl* 129 mice did not yield teratomas.

Although, testicular teratomas can only be induced in a limited number of strains, teratomas can also be formed from many strains of mice by the transplantation of rather earlier embryos, at the egg cylinder stage (about 7 days of development) to ectopic sites (Solter *et al.* 1970, 1979, 1981). As in the spontaneous ovarian tumours derived from parthenogenotes, these embryos become disorganized and form teratomas or teratocarcinomas, depending upon the host strain into which the embryo is transplanted and, interestingly, not upon the genotype of the embryo itself. Curiously, the range of mouse strains from which teratomas and teratocarcinomas can be derived by this route, and so from which EC cell lines can be established, is considerably greater than the range of strains from which ES cells have been derived by blastocyst culture, or teratocarcinomas from genital ridges, whether spontaneously or by transplantation. These results raise the question of what is the cell of origin of the EC cells derived from egg cylinder explants—presumably they are neither ICM/epiblast cells nor primordial germ cells. A further curiosity is that, apart from the spontaneous human tumours, it has not proved possible to derive teratocarcinomas routinely by such techniques in other species, though in the rat the yolk sac can give rise

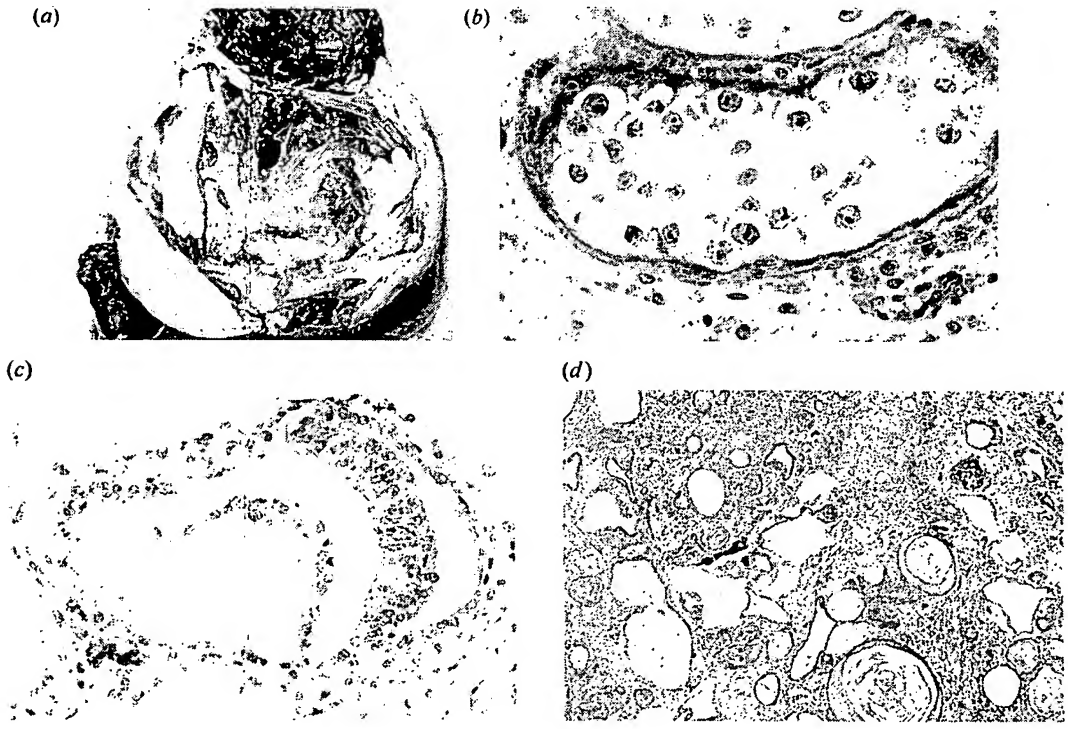

Figure 1. Examples of ovarian and testicular teratocarcinomas. (*a*) A mature human teratoma, or dermoid cyst of the ovary, showing hair and a tooth. (*b*) Carcinoma *in situ*: the pre-invasive phase of human testicular teratocarcinomas in which abnormal gonocytes fill the seminiferous tubules of the testis. (*c*) An embryoid body within a human testicular teratocarcinoma. (*d*) A well-differentiated mouse teratocarcinoma. Note extensive differentiation including keratin pearls and cartilage.

to a teratoma after the fetus is surgically removed (Sobis *et al.* 1993). These rat tumours are not re-transplantable and the isolation of a rat EC, or indeed ES, cell line has proved elusive.

3. EMBRYONAL CARCINOMA CELL LINES

Further study of the biology of mouse EC cells awaited the derivation of EC cell lines established in culture, which was first reported by Finch & Ephrussi (1967) and Kahn & Ephrussi (1970). Subsequently, several groups established murine EC cell lines throughout the 1970s (Bernstine *et al.* 1973; Jakob *et al.* 1973; Evans 1972; Martin & Evans 1974, 1975; Nicolas *et al.* 1975). Many of these lines remained pluripotent and, although they could be maintained as undifferentiated EC cells under certain culture conditions, they retained the ability to form teratocarcinomas when transplanted back into an appropriate mouse host. Many of the pluripotent lines would also differentiate in culture, but the circumstances varied between lines: some EC cell lines remained undifferentiated if kept proliferating in subconfluent cultures, but differentiated spontaneously to yield a variety of cell types, including nerve and muscle, if maintained in confluent cultures for several days (Nicolas *et al.* 1975). Other pluripotent EC lines required culture on feeder layers of transformed mouse fibroblasts to prevent differentiation (Martin & Evans 1974, 1975). In this case, differentiation could be induced by removing the EC cells from the feeder cells, and particularly if the cells were grown in suspension when they aggregated to form structures known as embryoid bodies with an inner core of EC cells surrounded by a layer of cells resembling the visceral endoderm of an early mouse conceptus. Gradually these embryoid bodies became histologically complex and a wide variety of differentiated cells would grow out when they plated on a substrate that permitted attachment.

The uncontrolled nature of spontaneous differentiation of EC cells, although dramatic, made study of the underlying processes difficult. The discovery by Strickland & Mahadavi (1978), that an apparently nullipotent EC cell line, F9, could be induced to differentiate by exposure to retinoic acid, proved a significant advance. After exposure to both retinoic acid and cAMP these F9 EC cells generated cells that closely resemble parietal endoderm (Strickland *et al.* 1980). Subsequently, it was found that if F9 cells are allowed to form clusters in suspension in the presence of retinoic acid, they form embryoid bodies in which the outer layer of cells resembles visceral endoderm, while the inner cells retained an EC phenotype (Hogan *et al.* 1983). Thus, apparently F9 cells can be switched between differentiating into visceral or parietal endoderm depending upon external cues. It was later shown that retinoic acid, as well as other agents, notably dimethylsulphoxide (DMSO) and hexamethylene bisacetamide (HMBA), could also induce the differentiation of a number of mouse EC cell lines (Jakob *et al.* 1978; McBurney *et al.* 1982). In particular, at least in the case of the P19 EC cell line, retinoic acid tended to induce neurectodermal differentiation, whereas DMSO induced mesoderm differentiation and the appearance of cardiac muscle (Jones-Villeneuve *et al.* 1982).

4. RELATIONSHIP TO THE EARLY EMBRYO

During the 1970s, it was found that murine EC cells express various features that are typical of cells of the ICM and primitive ectoderm of the early conceptus (Artzt *et al.* 1973; Jacob 1978). Following the line of reasoning that EC cells are a malignant equivalent of those embryonic cell types, EC cells from tumours were transplanted into blastocysts that were then re-implanted into the uterus of pseudo-pregnant female mice (Brinster 1974; Papaioannou *et al.* 1975; Mintz & Illmensee 1975). Under these conditions, the EC cells, which would typically form teratomas if transplanted to ectopic sites of an adult mouse, often appear to become normalized under the influence of the blastocyst and to participate in embryonic development giving rise to a range of normal tissues in the chimeric mice that subsequently develop.

In rare cases, it was reported that the germ cells of such chimeric mice also derived from the transplanted EC cells, but these observations have not been confirmed. Despite early indications to the contrary, it appears that many EC cells when transplanted into blastocysts do give rise to teratomas in the developing mice, so that their tumour characteristics are not completely suppressed by the host embryo. The latter result is, perhaps, not surprising as EC cells have generally adapted over many generations to growth either in culture or as a tumour in a host mouse. Because their differentiated derivatives usually have a limited lifespan and are non-malignant, one can easily imagine that the accumulation of mutations that inhibit differentiation would provide the EC cells with a selective advantage. When mouse EC cells are fused with somatic cells, notably thymocytes from adult mice, the resulting hybrid cells often exhibit the general features of EC cells (Miller & Ruddle 1976; McBurney 1977; Rousset *et al.* 1980; Gmür *et al.* 1980). However, in several cases, it has been noted that these hybrid EC cells show a greater capacity for differentiation than their EC parents (Andrews & Goodfellow 1980; Rousset *et al.* 1983). The simplest explanation for these observations is that the normal genome of the somatic cell introduces genes that are capable of complementing accumulated EC cell mutations that have tended to inhibit their ability to differentiate.

The recognition that EC cells are the malignant counterparts of embryonic ICM cells eventually resulted in the experiments of Evans & Kaufman (1981) and Martin (1981), who showed that it is possible to derive permanent lines of cells directly from mouse blastocysts, which closely resemble the EC cells derived from teratomas. They termed these cells ES cells. The normal cells to which these lines are thought to be equivalent, namely the cells of the late ICM, do not normally persist for any great length of time. The apparent ability of ES cells to grow indefinitely and exhibit an immortal characteristic, i.e. to present classical 'stem-cell features', seems to be a consequence of their removal from the embryo and maintenance in tissue culture. A further development, stimulated by the origins of testicular teratocarcinomas from primordial germ cells, was the finding that when such primordial germ cells are cultured *in vitro* they convert to cells, called embryonic germ (EG) cells, that closely resemble EC and ES cells (Matsui *et al.* 1992; Resnick *et al.* 1992).

5. HUMAN EC AND ES CELL LINES

Human teratocarcinoma cell lines were first isolated in the 1950s as xenografts in hamster cheek pouches (Pierce *et al.* 1957). Several lines were subsequently established *in vitro* during the 1970s, notably TERA1, TERA2 (Fogh & Trempe 1975) and SuSa (Hogan *et al.* 1977). Many of these human lines showed little capacity for differentiation, but they provided the basis for the identification of a number of characteristic features of human EC cells. Eventually, several human EC cell lines capable of differentiation were obtained, including: GCT27 (Pera *et al.* 1989; Roach *et al.* 1993, 1994; Pera & Herszfeld 1998), NCCIT (Teshima *et al.* 1988; Damjanov *et al.* 1993) and NCG.R3 (Hata *et al.* 1989; Umezawa *et al.* 1996) and, ironically, TERA2 (Andrews *et al.* 1984*b*), one of the oldest extant human teratocarcinoma cell lines. The pluripotent character of TERA2, and indeed its identity as an EC cell line, was overlooked for some time (e.g. see Andrews *et al.* 1980) because the specific culture conditions required to maintain pluripotent, undifferentiated human EC cells were not fully appreciated. Thus, in contrast to many mouse EC lines, it is necessary to maintain human EC cells at high cell densities, and it is generally best to passage TERA2 by scraping rather than by using trypsin to harvest the cells—retaining the cells in small clumps, which would be disrupted by trypsinization, seems to inhibit spontaneous differentiation. The widely used clonal subline of TERA2, NTERA2 cl.D1 (NT2/D1), was re-isolated after passage of a well-differentiated culture of TERA2 as a xenograft tumour in a nude mouse, which appeared to 'rescue' persisting EC cells within the culture (Andrews *et al.* 1984*b*).

Human and mouse EC cells differ significantly from one another, although they share some common features. For example, their morphology and growth patterns are similar, as both tend to grow in clusters of tightly packed cells with relatively little cytoplasm and prominent nucleoli. Also, both express high levels of alkaline phosphatase (Bernstine *et al.* 1973; Benham *et al.* 1981). However, the differences include a distinct pattern of surface antigen expression, as well as a propensity for human but not murine EC cells to differentiate into trophectoderm (Andrews *et al.* 1980, 1982, 1984*b*, 1996; Damjanov & Andrews 1983). The specific features of murine EC cells that differ from human EC cells, notably their expression of the lactoseries glycolipid antigen, stage-specific embryonic antigen 1 (SSEA1) (Kannagi *et al.* 1982; Gooi *et al.* 1981; Solter & Knowles 1978), their lack of expression of two globoseries glycolipid antigens, SSEA3 (Shevinsky *et al.* 1982) and SSEA4 (Kannagi *et al.* 1983), and their lack of ability to differentiate into trophectoderm, are all shared with murine ES cells, consistent with the idea that they resemble embryonic cells of the ICM and primitive ectoderm: although SSEA3 and SSEA4 are expressed by cleavage stage mouse embryos, the ICM lacks these antigens, but expresses SSEA1. Further, the ICM cells soon lose the capacity for trophectodermal differentiation. Thus, without the existence of human ES lines and without direct information from human embryos, the differences between human and mouse EC cells made the relationship of human EC cells to the early human embryo uncertain.

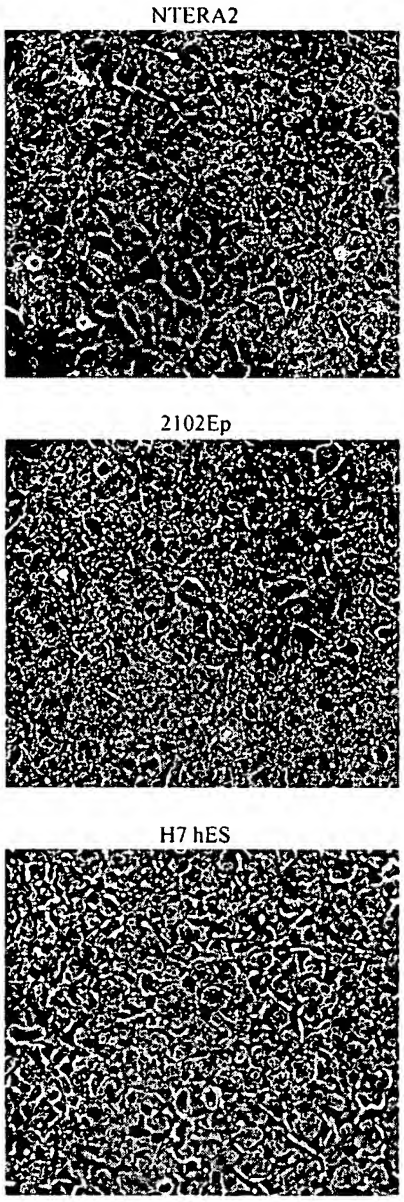

Figure 2. A comparison of the morphology of a human ES cell line, H7, provided by Dr James Thomson, and two human EC cell lines: NTERA2, capable of extensive differentiation (Andrews *et al.* 1984*b*), and 2102Ep, capable of only limited differentiation (Andrews *et al.* 1982). Note the tightly packed cells with little cytoplasm, pale nuclei and prominent nucleoli.

Logistical problems, as well as legal and ethical ones, delayed the derivation of ES cell lines from human embryos. However, rhesus monkey ES cell lines were first reported in 1995 (Thomson *et al.* 1995) and marmoset ES lines in 1996 (Thomson *et al.* 1996). These primate ES cells more closely resemble human EC cells than murine EC and ES cells. Eventually the derivation of human ES cell lines was reported by Thomson *et al.* (1998) and Reubinoff *et al.* (2000). These human ES cells also resemble the rhesus monkey ES cells and human EC cells (figure 2) rather than the murine cells—they are able to differentiate into trophoblast and are SSEA3(+), SSEA4(+), SSEA1(−). Also like human EC cells, but not those of the mouse, they express class 1 MHC antigens and Thy1 (Draper *et al.* 2002). Conversely, human ES cells do resemble the mouse cells in their expression of alkaline phosphatase and the stem-cell-specific transcription factor, *Oct4* (Yeom *et al.* 1996). Recently, we have confirmed that indeed the ICM of human blastocysts resembles human EC and ES cells in several of these features (Henderson *et al.* 2002), notably the expression of SSEA3 and SSEA4, the lack of SSEA1 and also the expression of two human cell-surface, keratan sulphate-related antigens, TRA-1-60 and TRA-1-81, which were previously defined on human EC cells (Andrews *et al.* 1984*a*; Badcock *et al.* 1999). Thus, there do appear to be significant differences in the biology of early embryonic cells of human and mouse embryos.

The precise significance of these differences remains to be resolved, especially as the function of the surface antigen markers, mostly involving carbohydrate epitopes, is unknown. Nevertheless, the regulation of these molecules is subject to tight control during differentiation; SSEA1 has been suggested to mediate compaction in mouse embryos (Bird & Kimber 1984; Fenderson *et al.* 1984), and a role for SSEA3 and SSEA4 in embryo pathology has been suggested (Tippet *et al.* 1986). SSEA3 and SSEA4 belong to the P-blood-group system and are expressed on red cells, except in individuals with the pp or p^k phenotypes. Women with these latter phenotypes are reported to be susceptible to recurrent early spontaneous abortions (Race & Sanger 1975); the expression of SSEA3 and SSEA4 by early embryonic cells suggests that these could be the target for a proposed immune response to the early embryos in such women. However, regardless of the functional significance of these particular differences between mouse and human embryos, the results show that lessons from the mouse cannot necessarily be extrapolated to human embryogenesis. They also suggest that human EC cells remain a useful complement of human ES cells for exploring the mechanisms that regulate human development; while ES cells are 'genetically normal' and differentiate into a wide range of cell types, EC cells are easier to grow and exhibit a simplified pattern that can be analysed more easily.

Human EG cells have also been described but, although pluripotent, they seem to differ from human EC and ES cells with respect to various surface antigen markers (Shamblott *et al.* 1998). However, the significance of these differences remains to be resolved.

6. DIFFERENTIATION OF HUMAN EC AND ES CELLS

Although EC cells are the stem cells of teratocarcinomas, a striking feature of many human EC cell lines is their lack of ability to differentiate into well-recognizable cell types. This might, in part, reflect their origins in tumours, as the acquisition of an inability to differentiate could provide a strong selective advantage. Human EC cells are highly aneuploid and it is easy to envisage that genetic changes might occur to inhibit their differentiation. Indeed, cell-hybrid studies with both mouse and human EC cells support this idea and suggest that a loss of pluripotency results from the loss of key gene functions

(Andrews & Goodfellow 1980; Duran *et al.* 2001). Nevertheless, several human EC cell lines can differentiate well and have been studied extensively. One line, GCT27, requires maintenance on feeder layers and differentiates into a wide range of cell types when removed from the feeders (Pera *et al.* 1989). Another human EC cell line, TERA2 and its NTERA2 sublines, does not require feeders and differentiates extensively, but in distinct directions, in response to retinoic acid (Andrews 1984), HMBA (Andrews *et al.* 1990) and the bone morphogenetic proteins (BMPs) (Andrews *et al.* 1994).

Apart from forming well-differentiated teratomas when grown as xenografts in nude mice, TERA2 and NTERA2 cells rapidly lose their EC phenotype and differentiate into a wide array of cell types, including neurons, after exposure to 10^{-5} or 10^{-6} M retinoic acid (Andrews 1984). This differentiation is marked by a switch in glycolipid synthesis from globoseries to lactoseries and ganglioseries structures (Fenderson *et al.* 1987), and can conveniently be followed by the loss of antigens, such as SSEA3 and SSEA4, as well as TRA-1-60 and TRA-1-81, and the acquisition of antigens, such as SSEA1, A2B5 (ganglioside GT3) and ME311 (ganglioside 9-*O*-acetyl GD3). There are also substantial changes in gene activity, most notably activation of the *HOX* genes in a retinoic acid concentration-dependent manner (Simeone *et al.* 1990), and the appearance of susceptibility to infection and replication of human cytomegalovirus (Gönczöl *et al.* 1984) and human immunodeficiency virus (Hirka *et al.* 1991), neither of which will grow in the undifferentiated EC cells.

As differentiation progresses, neural markers become evident, and neurons that express neurofilament proteins and a typically neuronal morphology appear (figure 3*a*) after one to two weeks (Andrews 1984; Lee & Andrews 1986). The pattern of expression of genes related to the early stages of neural differentiation during embryonic development—nestin in mitotic neural precursors and NeuroD in post-mitotic neuroblasts—seems to be followed by differentiating NTERA2 cells (Przyborski *et al.* 2000; Pleasure *et al.* 1992; Pleasure & Lee 1993). Furthermore, the terminal neurons are functional and express tetrodotoxin-sensitive sodium channels (Rendt *et al.* 1989), glutamate receptors and voltage-gated calcium channels (Squires *et al.* 1996). Several studies have recently been conducted of their potential for implanting into the central nervous system, initially in experimental rats in which NTERA2-derived neurons will apparently integrate functionally to correct neural defects, such as those resulting from stroke (Borlongan *et al.* 1998; Hurlebert *et al.* 1999; Kleppner *et al.* 1995; Philips *et al.* 1999). Experiments to implant these EC-cell-derived neurons into human stroke patients have also been reported (Kondziolka *et al.* 2000). The tumour origins of these neurons provokes some disquiet about safety, and whether their implantation will provide benefits to patients remains to be seen. Nevertheless, the experiments pave the way for future studies using neurons derived from genetically normal ES cells.

When differentiation of NTERA2 EC cells is induced by HMBA or BMP7, distinct differences from retinoic-acid-induced differentiation are seen (Andrews *et al.* 1990, 1994). Thus, ganglioside antigens are only expressed late, on a small proportion of cells, and few neurons are evident. The identity of many of the cells induced by HMBA and BMP has not been clearly defined, although smooth muscle actin was particularly noted after BMP induction. One idea we have considered is that NTERA2 differentiates predominantly in an ectodermal direction but that, depending upon the conditions, this can take a more 'dorsal' (predominantly neural) or 'ventral' (epidermal) character. By this notion, retinoic acid may favour a more dorsal pattern of differentiation, whereas HMBA and BMP may promote a more ventral pattern.

Not surprisingly, the embryo-derived human ES cells exhibit a considerably greater potential for differentiation. When grown in immunodeficient mice, these cells form well-differentiated teratomas with well-organized tissues representing all three germ layers (Thomson *et al.* 1998). When grown *in vitro*, as for mouse ES cells, the human ES cells require maintenance on feeder layers to prevent differentiation, but the cytokine LIF, which is able to prevent the differentiation of mouse ES cells in the absence of feeders, is apparently not able to do so for human ES cells. Conversely, it has been reported that conditioned medium from fibroblasts will inhibit differentiation of cells grown on Matrigel (Xu *et al.* 2001).

Many studies of differentiation in human ES cell cultures have focused upon allowing the cells first to form 'embryoid bodies' by culture in suspension; after a period of suspension culture, a variety of differentiated cells can be detected in the resulting cell clusters, which will grow out when allowed to attach. Under these conditions, neurons, glia, skeletal and cardiac muscle, liver and even insulin-secreting islet cells have been reported (Assady *et al.* 2001; Kaufman *et al.* 2001; Kehat *et al.* 2001; Itskovitz-Eldor *et al.* 2000; Odorico *et al.* 2001; Pera 2001; Schuldiner *et al.* 2000).

Differentiation also occurs in attached cultures, either spontaneously, or by induction with agents such as retinoic acid. Even when maintained on feeders, colonies of clearly non-ES cells can often be seen, and gene expression indicative of extra-embryonic cell types (e.g. chorionic-gonadotropin-indicating trophoblast and α-fetoprotein-indicating yolk sac) can be detected in the SSEA3(−) presumptive non-ES cells isolated by 'fluorescence-activated cell sorting' from such cultures (Henderson *et al.* 2002). These spontaneous differentiated derivatives can present a problem to continued maintenance of the ES lines if they are allowed proliferate, and culture techniques need to be adapted to remove them during passaging.

Retinoic acid, HMBA and DMSO also induce differentiation of attached cultures, whether in the presence or absence of feeders (Draper *et al.* 2002). Many of the changes seen during the differentiation of human EC cells induced by these agents are also seen in the human ES cultures. For example, all the typical EC/ES marker antigens, SSEA3, SSEA4, TRA-1-60, TRA-1-81, etc. are downregulated. Also, neural differentiation is common (figure 3*b*). However, although the induction of some of the antigens that appear during EC differentiation is seen, there are many differences in the patterns of antigens observed, probably because the ES cells are capable of a much wider range of differentiation.

Although it is clear that human ES cells are capable of extensive differentiation, as in the mouse, and although

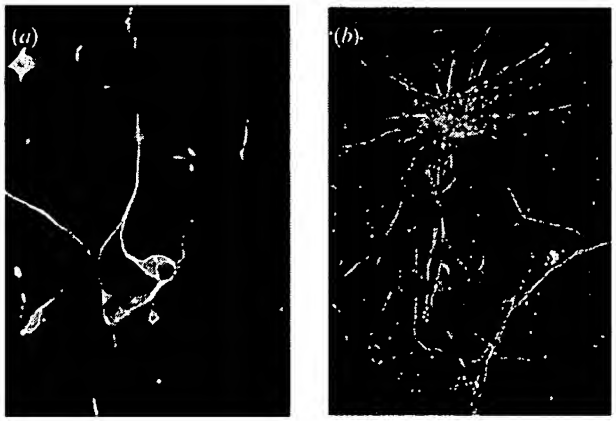

Figure 3. (*a*) Neurons differentiating from NTERA2 human EC cells induced with retinoic acid, and (*b*) a low-power view of an extended network of neurons in a culture of human ES cells (H7), also induced with retinoic acid. Both are stained with an antibody to the 200 kDa neurofilament protein.

various protocols are being developed to favour particular lineages of differentiation, we currently know very little of the mechanisms that regulate their decision to differentiate in the first place, or that regulate which lineages are followed once differentiation is initiated. Answers to these fundamental questions could provide valuable insights into a wide range of issues pertinent to human health, from the causes of abnormal embryonic development and the formation of birth defects, to understanding the relationships between cell proliferation and differentiation that underlie the development of cancer. Such answers are also crucial if ES cells are to realize their promise of providing tissues for transplantation and 'regenerative' medicine. Thus, efficient maintenance of ES cells suitable for use in human patients will demand culture conditions that minimize not only spontaneous, unwanted differentiation, but also the use of animal products, whether feeder cells, serum or growth supplements, which could introduce adventitious pathogens. Equally, although a wide range of differentiation is easy to obtain, particular cell types often only constitute a small proportion of a differentiated culture. Efficiency of production, apart from the need to avoid contamination with unwanted cell types that may be deleterious, demands that culture techniques are developed to promote differentiation in only specific directions.

Although murine EC and ES cells were originally derived with approaches to addressing such questions in mind, most of the use of mouse ES cell technology over the past 20 years has been directed towards production of transgenic mice, and not for answering questions of fundamental cell biology pertinent to ES cells *per se*. Nevertheless, some studies of the mechanisms that regulate mouse ES cell differentiation have been carried out. Most notable are studies that have highlighted the crucial role of specific discrete levels of *Oct4* expression for the maintenance of an undifferentiated phenotype (Niwa *et al.* 2000). Thus, if the level of *Oct4* expression in mouse ES cells is reduced, differentiation into trophectoderm ensues, whereas if levels are raised, differentiation to extra-embryonic endoderm is promoted. Furthermore, the maintenance of an undifferentiated phenotype depends upon activation of the STAT3 signalling pathway and inhibition of the MAPK/ERK pathway, following interaction of LIF with its receptor (Niwa *et al.* 1998; Burdon *et al.* 1999; Boeuf *et al.* 1997). However, as LIF does not appear to influence the behaviour of human ES cells (Thomson *et al.* 1998; Reubinoff *et al.* 2000), it remains uncertain to what extent these lessons will apply to human ES cells. It is possible that the STAT3 and MAPK pathways could be regulated by other receptor ligand systems; alternatively, other signalling pathways, yet to be identified, might perform similar functions in humans.

7. CONCLUDING THOUGHTS

A common notion that has prevailed in developmental biology for many years is one of cell differentiation during embryogenesis proceeding through a series of successive binary decisions by which cells adopt alternative phenotypes. Thus, embryogenesis is commonly seen in terms of cells following branching pathways of differentiation. Waddington (1956, 1966) has described such a process as 'canalization', envisaging cells moving 'downhill' through a series of valleys making up the 'epigenetic landscape' (figure 4*a*). A further notion that is often linked to this concept, although by no means essential, is that the process of differentiation is unidirectional and, in normal circumstances, irreversible. The branching pathways are seen as representing successive commitment of cells progressively to restricted options of eventual cell fate.

While the idea of 'de-differentiation', implying reversal of specific steps of differentiation, has often been discussed, particularly in the context of tumour biology, it has not found general favour. In the case of cancers that appear, for example, to express so-called 'oncofetal' proteins, it may be more appropriate to consider that it is the stem cells of specific tissues that are the target for carcinogenesis, and that it is their overgrowth that gives the appearance of reversion, or 'de-differentiation' (Pierce 1974). However, the recent reports of stem cells from different adult tissue, displaying quite unexpected plasticity and apparent lack of specific commitment, suggests that perhaps the concepts of unidirectional, irreversible differentiation along distinct cell lineages should be revised. Indeed, 'trans-differentiation', as in metaplasia, is well known to pathologists, while 'trans-determination' of imaginal disc cells in *Drosophila* was described many years ago by Hadorn (1968).

When considering the factors that regulate cell behaviour, whether commitment and determination, or differentiation, attention commonly focuses on individual signalling pathways by which cells respond to external cues, e.g. growth factors, the extracellular matrix, or interactions with other cells. To keep the analysis simple, such signalling pathways within a cell are often considered in isolation, and are also considered as simple switches—either 'on' or 'off'. However, any molecules within a signalling pathway will obey the normal chemical laws affecting reaction rates and equilibria. The activity of particular regulatory molecules will be influenced by the overall state of all the other regulatory and metabolic reactions taking place within the cell. Thus, the various signalling and

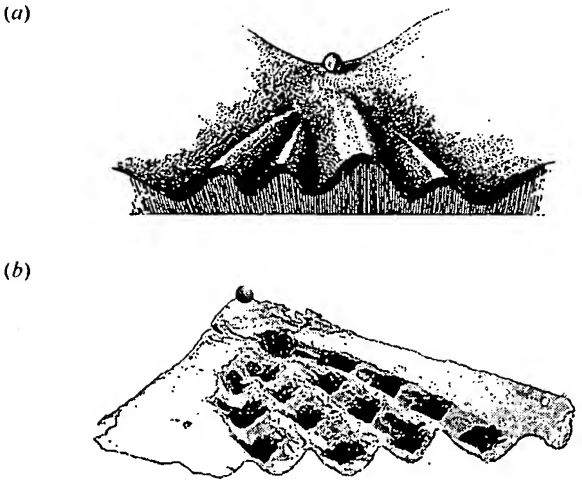

Figure 4. The 'epigenetic landscape': (*a*) a diagram reproduced from a drawing by C. H. Waddington (Waddington 1966) to illustrate his concept of 'canalization'. The image of a ball rolling downhill and selecting between different valleys represents the selection of different lineages by embryonic cells as they differentiate; if something were to push the ball up the side of a valley, it would nevertheless tend to return to the valley bottom, effectively a representation of regulation within the developing embryo. (*b*) A modification of the idea of Waddington: in this image the 'landscape' includes a series of depressions or hollows, which may nevertheless be arranged in valleys. The hollows would represent lowest free energy states, i.e. stable states, of the regulatory/metabolic network that makes up a cell. Movement of a cell from one state to another (differentiation) would tend to be from a stable state of higher free energy to one of lower free energy: the likelihood of such a transition would depend upon the height of the surrounding terrain—low barriers, say between successive hollows in a given valley, would permit a high frequency, or probability, of transition, whereas high barriers, say between neighbouring valleys, would permit transitions only infrequently. Thus, the height of the ground represents something akin to activation energy.

regulatory pathways, as well as the metabolic pathways of a cell, make up a complex, dynamic network in which various 'stable states' or equilibria would probably exist. These stable states, which would correspond to free energy minima for the network, would represent the various differentiated states that a cell could adopt. Under this view, it may be appropriate to modify the 'valleys' envisaged in Waddington's epigenetic landscape to a series of depressions (figure 4*b*), possibly still linked in 'valley systems', that would represent the commonly observed differentiation pathways. The height of the terrain would represent the free energy of the regulatory/metabolic network that constitutes the cells. Therefore the probability of a cell moving from one state to another would depend upon the heights of the hills surrounding the hollows and valleys. Cells finding themselves on the 'uplands' of 'unstable' or 'transition' states would tend to move to the nearest 'stable' state. In such a model, normally observed lineages represent transitions for which there is the highest probability of movement, hence the appearance of irreversibility. However, reversion, or de-differentiation, is entirely possible, although the probability may be low, as would movements from one set of hollows within one valley system to another valley system—trans-differentiation or trans-determination.

Waddington (1962) has indeed discussed the concept of stable states for cells and referred to mathematical models developed by Goodwin (1961). Slack (1991) has also discussed a similar idea. Perhaps a substantial challenge from the recent observations of stem-cell plasticity will be to develop a more detailed understanding of the chemistry underlying the complex network of signalling and metabolic pathways within a cell, to provide a more solid basis for such a concept of 'stable states' as opposed to fixed cell lineages. Certainly, considerable work lies ahead to understand the molecular mechanisms that regulate stem-cell differentiation, before their full potential for regenerative medicine can be realized.

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REVIEW

Surface antigens of human embryonic stem cells: changes upon differentiation in culture*

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Abstract

We have analysed the surface antigen phenotype of a human embryonic stem (hES) cell line (H7) and the changes that occur upon differentiation induced by retinoic acid, hexamethylene bisacetamide and dimethylsulphoxide. The undifferentiated stem cells expressed Stage Specific Embryonic Antigen-3 (SSEA3), SSEA4, TRA-1-60, and TRA-1-8 but not SSEA1. In these characteristics they closely resemble human embryonal carcinoma (EC) cells derived from testicular teratocarcinomas, and are distinct from murine EC and ES cells. The undifferentiated cells also expressed the liver/bone/kidney isozyme of alkaline phosphatase detected by antibody TRA-2-54, the class 1 major histocompatability antigens, HLA-ABC, and the human Thy1 antigen. Differentiation of hES cells was induced by retinoic acid, HMBA and DMSO with the appearance of various cell types including neurons and muscle cells. The surface antigens characteristically expressed by hES cells were down-regulated following induction of differentiation and other antigens appeared, notably several ganglioside glycolipids detected by antibodies VIN-IS-56 (GD3 and GD2), VIN-2PB-22 (GD2), A2B5 (GT3) and ME311 (9-O-acetyl-GD3). Whereas the expression of HLA was slightly down-regulated upon differentiation, its expression was strongly induced by interferon-γ in both the undifferentiated and the differentiated cells, although the induction in the differentiated cultures was considerably stronger than in the stem cells. In all of these features the human ES cells, and their pattern of differentiation, resembled the pluripotent human EC cell line NTERA-2 although clearly the range of cells generated by the hES cells was considerably greater.

Key words stem cells; surface antigens.

Introduction

EC cells are the malignant stem cells of teratocarcinomas, a subset of germ cell tumours that may contain many embryonic and extra-embryonic tissues, and apparently recapitulate many aspects of cell differentiation that occur during normal embryogenesis, albeit in a disorganized manner. Both spontaneous and experimentally induced teratocarcinomas occur in the laboratory mouse (Solter & Damjanov, 1979). By now it is well documented that murine EC cells isolated from such teratocarcinomas closely resemble cells of the inner cell mass from the blastocyst stage of the early mouse embryo, and embryonic stem (ES) cells cultured directly from explanted blastocysts (Martin, 1980). For example, when mouse EC and ES cells are placed into a blastocyst they can participate in embryonic development, and contribute to normal tissues in the resulting chimeric embryo (Brinster, 1974; Papaioannou et al. 1975). ES cells form chimeras much more efficiently than EC cells, but this may well reflect the accumulation of genetic and epigenetic changes that occur in EC cells as they adapt to efficient tumour growth.

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Until the recent derivation of human ES cell lines, human EC cell lines provided the best model for studying cell differentiation pertinent to human embryonic development (Andrews, 1998). For example, the human teratocarcinoma-derived cell line NTERA2 is composed of pluripotent EC cells that differentiate in response to retinoic acid, yielding a variety of cell types including functional, post-mitotic neurons (Andrews et al. 1984b; Andrews, 1984; Rendt et al. 1989; Pleasure et al. 1992; Squires et al. 1996). This differentiation is characterized by marked changes in gene expression (Ackerman et al. 1994) but most notably by the activation of HOX genes in a retinoic acid concentration-dependent manner (Simeone et al. 1990). However, changes in the expression of cell surface antigens provide a particularly powerful means of monitoring differentiation, not only because antigen expression can be readily assessed on single cells in complex differentiating populations, but because subsets of viable cells corresponding to different stages of differentiation, or different lineages, can be isolated in order to analyse their individual properties and capacity for further differentiation (Fenderson et al. 1987).

Human EC cells, like NTERA2, are characterized by expression of the globoseries glycolipid antigens SSEA3 and SSEA4, and by a lack expression of a lactoseries oligosaccharide antigen, SSEA1 (Andrews et al. 1982, 1984b, 1996). They also express the keratan sulphate-related antigens, TRA-1-60 and TRA-1-81 (Andrews et al. 1984a; Badcock et al. 1999) and the tissue non-specific alkaline phosphatase-related antigens TRA-2-49 and TRA-2-54 (Andrews et al. 1984c). Differentiation induced by retinoic acid is marked by the down-regulation of all of these antigens, and by the appearance of other antigens including SSEA1 and several ganglioseries glycolipid antigens, which segregate on different subsets of cells (Fenderson et al. 1987). If differentiation is induced by another agent, hexamethylene bisacetamide (HMBA), the EC-specific antigens are similarly down-regulated, but the antigens induced by retinoic acid do not appear, consistent with the notion that retinoic acid and HMBA induce differentiation along distinct lineages (Andrews et al. 1990).

The recent derivation of ES cell lines from human embryos (Thomson et al. 1998; Reubinoff et al. 2000) now provides additional tools for investigating the molecular processes that guide human development and the causes of infertility and birth defects when these mechanisms operate incorrectly. Further, human ES cells could provide a potential source of 'normal' differentiated cell types for transplantation therapies and drug discovery.

Initial studies have confirmed the anticipated similarities between human ES and EC cells, and significant differences in antigen expression and other properties between human ES and EC cells and their murine counterparts (Andrews, 1998). We have now examined the changes in surface antigen expression that occur during the differentiation of human ES cells induced by retinoic acid, HMBA or DMSO, or by culture in the absence of feeders. As anticipated, a number of changes that occur during EC cell differentiation, notably down-regulation of stem-cell-specific markers, also occurred during the differentiation of ES cells. There were also some differences in the patterns of antigens induced, mostly likely because of the greater diversity of differentiated cell types that appear during ES cell differentiation.

Materials and methods

Cell culture

The H7 human ES (hES) cell line, described by Thomson et al. (1998), was cultured on mouse embryonic fibroblast (MEF) feeder cells, mitotically inactivated using Mitomycin-C, as previously described. However, the cells were cultured in DMEM-SR medium (Gibco-BRL) supplemented with 4 ng mL^{-1} bFGF (Gibco-BRL) and 20% 'Serum Replacement' (SR) (Gibco-BRL) instead of fetal calf serum. Briefly, MEFs isolated from 13-day mouse embryos (Robertson, 1987) were treated with 10 $\mu g\ mL^{-1}$ mitomycin-C (Sigma Aldrich) for 2.5 h. Subsequently, the treated cells were washed with PBS, harvested with trypsin-EDTA and reseeded at 10^4 cells per cm^2 on gelatin-treated tissue culture dishes. For passaging, the H7 hES cells were treated with 1 mg mL^{-1} Collagenase type IV (Sigma Aldrich) in DMEM:F12 for 8–10 min at 37 °C and then detached by scraping using glass beads (Andrews et al. 1984b), washed by centrifugation, and re-plated onto inactivated MEFs. In some experiments, H7 hES cells were plated on tissue culture dishes that had been pretreated with poly D-lysine and Matrigel (Becton-Dickinson) in the absence of inactivated MEFs. Differentiation was induced by adding 10^{-5} M all *trans*-retinoic acid (RA) (Eastman Kodak), 3 mM hexamethylene bisacetamide (HMBA) (Sigma Aldrich), or 1% dimethylsulphoxide (DMSO) as described by Andrews

(1984), Andrews et al. (1990) and Andrews et al. (1986), respectively. Human interferon-γ (IFNγ) was purchased from Sigma Aldrich.

NTERA2 cL.D1 human EC cells (Andrews et al. 1984b) were cultured as previously described and induced to differentiate by treatment with 10^{-5} M all-*trans* retinoic acid (RA) (Eastman Kodak) (Andrews, 1984).

Surface antigen expression

Cell surface antigen expression was assessed by immunofluorescence detected by flow cytofluorimetry after harvesting cultures as single cell suspensions using trypsin-EDTA, as previously described (Andrews et al. 1987a, 1987b; Fenderson et al. 1987). The following monoclonal antibodies were used to detect surface antigen expression: MC631, anti-Stage Specific Embryonic Antigen-3 (SSEA3) (Shevinsky et al. 1982), MC813-70, anti-Stage Specific Embryonic Antigen-4 (SSEA4) (Kannagi et al. 1983), MC480, anti-Stage Specific Embryonic Antigen-1 (SSEA1) (Solter & Knowles, 1978), TRA-1-60 and TRA-1-81 (Andrews et al. 1984a), TRA-2-54, anti-liver/kidney/bone alkaline phosphatase (Andrews et al. 1984c), A2B5 (Eisenbarth et al. 1979; Fenderson et al. 1987), ME311 (Thurin et al. 1985; Fenderson et al. 1987), VIN-IS-56 and VIN-2B-22 (Andrews et al. 1990), N901 (Griffin et al. 1983) and anti-Thy1 (McKenzie & Fabre, 1981), W6/32 (Barnstable et al. 1978), BBM1 (Brodsky et al. 1979). TRA-1-85 (Williams et al. 1988), which detects a pan-human antigen, was used to monitor contamination with mouse feeder cells.

Results

Cultures of H7 hES cells grown on mouse embryo fibroblast feeders contained many colonies of cells that closely resembled human EC cells (Fig. 1). If the cells were plated onto tissue culture dishes precoated with poly D-lysine and Matrigel, but without MEFs, many of the cells appeared to differentiate and many colonies of cells with markedly different morphologies appeared, although occasional colonies of cells retaining an ES phenotype persisted. If all-*trans*-retinoic acid was included into the cultures, either grown on feeder layers, or after plating on Matrigel, even more marked differentiation occurred and a variety of cell types, for example neurons, glia and muscle cells, could be detected by immunofluorescence staining with appropriate antibodies (Fig. 2).

We then examined the expression of a panel of antigens previously analysed on human EC cells (Fig. 3). As anticipated from previous reports, the hES cells from stock cultures, at the time of reseeding, strongly expressed the markers that also characterize undifferentiated EC cells, namely SSEA3, SSEA4, TRA-1-60 and TRA-1-81. They also strongly expressed Thy1, and the liver/bone/kidney isozyme of alkaline phosphatase detected by reactivity with antibody TRA-2-54. By contrast, they expressed relatively low levels of SSEA1. These characteristics are typical of the surface antigen phenotype of human EC cells and distinct in certain respects from murine EC cells. The proportion of feeder cells at the time of assay, monitored by reactivity with

Fig. 1 Phase contrast photomicrograph of an undifferentiated human ES cell colony. The cells resembled human EC cells morphologically, forming tight colonies composed of cells with high nucleus/cytoplasm ratios and containing few prominent nucleoli. Scale bar = 50 μm.

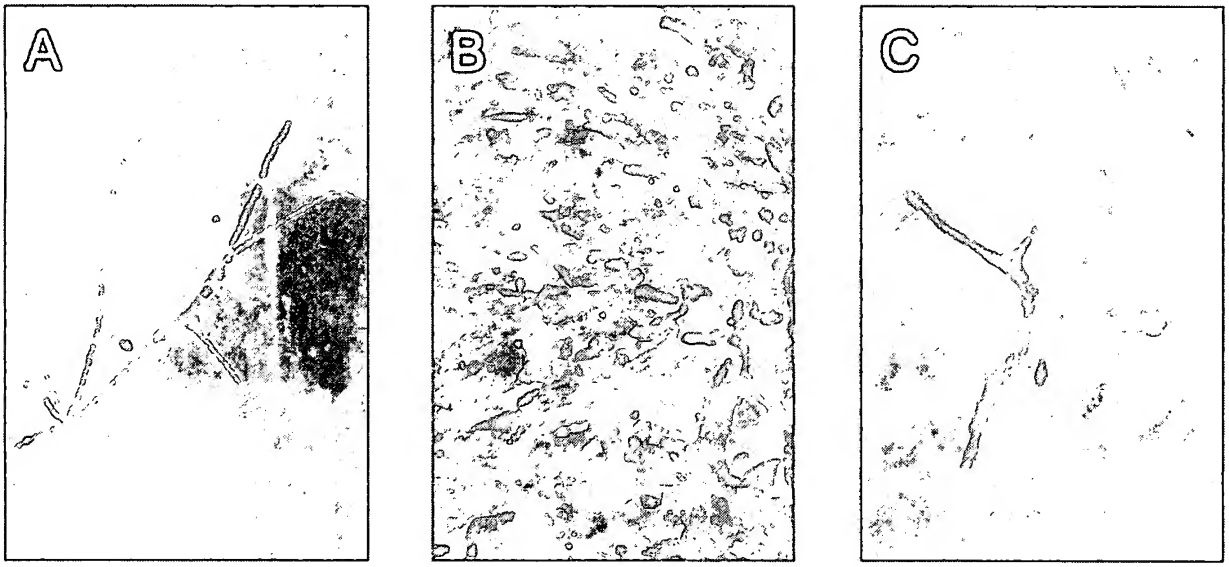

Fig. 2 Neurons, glial and muscle cells detected by immunofluorescence with antibodies to neurofilaments (A), GFAP (B) and desmin (C) were found in the hES H7 cultures grown in the presence of 10^{-5} M retinoic acid.

an antibody to a pan-human antigen, TRA-1-85, was negligible (< 2%) (data not shown).

During subsequent culture, expression of SSEA3, SSEA4, TRA-1-60, TRA-1-81, TRA-2-54 and Thy1 remained high when the ES cells were cultured on MEFs in the absence of retinoic acid. However, these antigens were down-regulated when the cells were plated on Matrigel in the absence of MEFs, or when they were cultured in the presence of retinoic acid, whether on MEFs or on Matrigel. Such changes are consistent with the differentiation of the cells, as suggested by morphological examination, and are comparable to changes seen during the differentiation of human EC cells.

The kinetics of disappearance of these antigens differed from one another. In particular, SSEA3 disappeared more quickly than SSEA4, a result which we have consistently found before in the case of differentiating human EC cells (Fenderson et al. 1987). TRA-1-60 and TRA-1-81 also disappeared rapidly, whereas TRA-2-54 and Thy1 tended to disappear relatively slowly. Whether this reflects persistent expression on a subset of differentiating cells or a slower turnover of the antigens was not assessed. A further notable point was that although the EC/ES markers were down-regulated when the cells were cultured on Matrigel in the absence of retinoic acid, a significant proportion of cells that continued expressing these antigens nevertheless persisted, suggesting that at least during short-term culture the presence of MEFs is not an absolute requirement for maintenance of an undifferentiated phenotype.

Differentiation of the NTERA2 EC cell line is marked by transient up-regulation of SSEA1 and a strong up-regulation of gangliosides, notably GT3 detected by A2B5, all consistent with a marked propensity of these cells to differentiate in a neuroectodermal direction (Fenderson et al. 1987). In the case of hES cells, the gangliosides GD3 and GD2 detected by antibodies VIN-IS-56 and VIN-2PB-22 were also up-regulated when the cells were cultured either without feeders or after addition of retinoic acid, but only relatively small numbers of cells expressed antigens detected by A2B5 (GT3) and ME311 (9-O-acetyl-GD3). In fact the greatest induction of these antigens appeared to occur without retinoic acid, when the cells were plated on Matrigel. SSEA1(+) cells were seen after culture on Matrigel and, even more after the addition of retinoic acid, although their numbers subsequently declined. The antigen detected by antibody N901, CD56, a version of NCAM, was up-regulated after addition of retinoic acid and to some extent after culture on Matrigel in the absence of retinoic acid.

The presence or absence of bFGF in the medium during differentiation had no significant effect on the pattern of antigen expression (data not shown).

The class 1 Major Histocompatibility Complex (MHC) antigens (HLA) are commonly expressed by human EC cells, in contrast to their absence from murine EC and

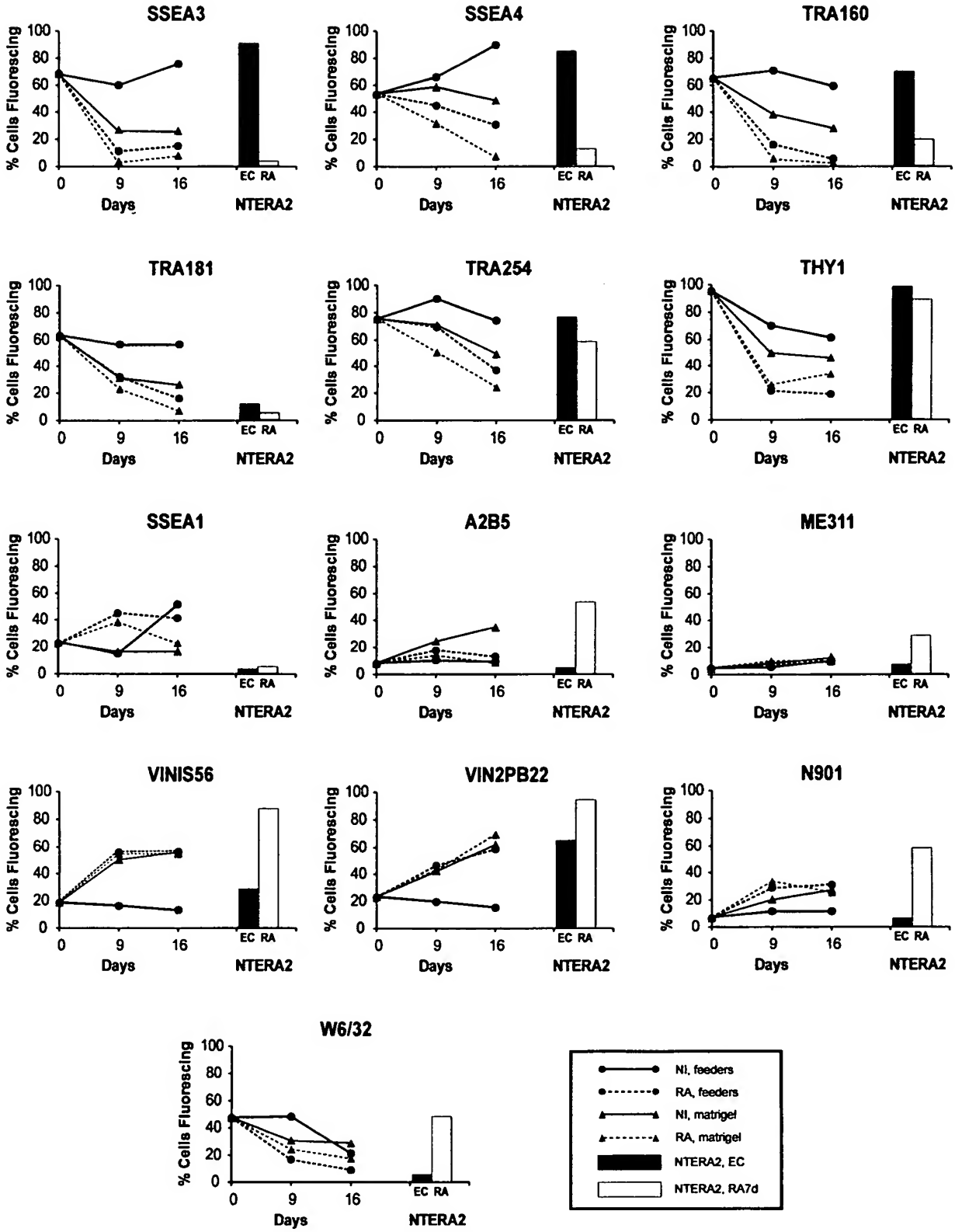

Fig. 3 Changes in surface antigen expression by hES H7 cells when cultured on MEF feeders or Matrigel, in the presence or absence of 10^{-5} M retinoic acid. For comparison typical antigen expression by NTERA2 EC cells and NTERA2 cells induced to differentiate with retinoic acid for 7 days are also shown.

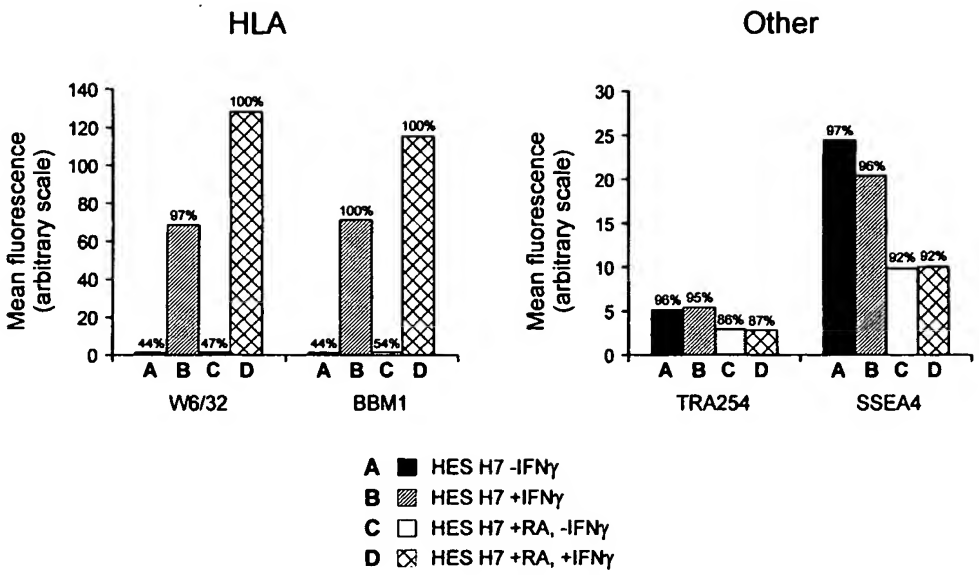

Fig. 4 The effect of IFNγ on the expression of HLA (HLA-A,B,C and β_2 microglobulin) and the antigens (alkaline phosphatase and SSEA4) by hES H7 cells. The histograms show the mean fluorescence intensity of cells after staining with antibodies W6/32 (anti HLA-A,B,C), BBM1 (anti-β_2 microglobulin), TRA-2-54 (anti-liver/bone/kidney alkaline phosphatase) and MCX813-70 (anti-SSEA4); the percentage of cells fluorescing is indicated above each bar.

ES cells (Andrews, 1998). HLA-A, B, C, the class 1 MHC antigen detected by antibody W6/32, was expressed by the undifferentiated H7 hES cells (Fig. 3), and some down-regulation was noted after differentiation induced with retinoic acid or on Matrigel or indeed on extended cultures. In human EC cells, HLA is strongly inducible by IFNγ without any obvious effects on differentiation, and is inducible to a higher level after differentiation. We have now found that the same is true of the H7 hES cells (Fig. 4). Thus, HLA-A, B, C, detected by W6/32, and β2 microglobulin, detected by antibody BBM1, were both much more strongly expressed after culture of the cells for 3 days in the presence of IFNγ. However, considerably stronger expression was noted on cells that had been pretreated with retinoic acid. The effect was specific to HLA; the expression of other surface antigens, for example, TRA-2-54 and SSEA4, was not affected by culture in the presence of IFNγ suggesting that there was no effect on the state of differentiation of the cells.

Although retinoic acid is, perhaps, the most widely used agent to induce differentiation of EC cells and ES cells, in both the mouse and human, other agents are also known to cause undifferentiated pluripotent cells to differentiate. Notably, HMBA induces differentiation of mouse (Jakob et al. 1978) and human EC cells (Andrews et al. 1990), while DMSO which induces differentiation of mouse EC cells (McBurney et al. 1982) has very little effect on, at least, the NTERA-2 human EC cell line (Andrews et al. 1986). When H7 hES cells were cultured in the presence of 1% DMSO or 3 mM HMBA (Fig. 5) after plating on Matrigel, without MEFs, significant differentiation was indicated by changes in antigen expression. All the antigens characteristic of human EC and ES cells, SSEA3, SSEA4, TRA-1-60, TRA-1-81, TRA 2–54 and Thy1, were strongly down-regulated in cultures exposed to the DMSO and HMBA. Again, SSEA3 disappeared rather more rapidly than SSEA4. At the same time antigens detected by antibodies A2B5, ME311 and N901 were also all induced by both DMSO and HMBA. Indeed, A2B5 reactivity appeared to be induced to a greater extent by DMSO than by retinoic acid. Neither agent induced SSEA1 to any significant extent. Thus, as with EC cells, HMBA and DMSO appear to induce differentiation along lineages to some extent distinct from those induced by retinoic acid.

Discussion

It has not always been evident that EC and ES cells in humans are related cell types. Although in the laboratory mouse EC cells do closely resemble ES cells, and may be considered their malignant counterparts, human EC cells differ in many respects from murine EC and ES cells. Thus the cell surface antigen phenotype of human EC cells is typically SSEA1(–) but SSEA3(+) and

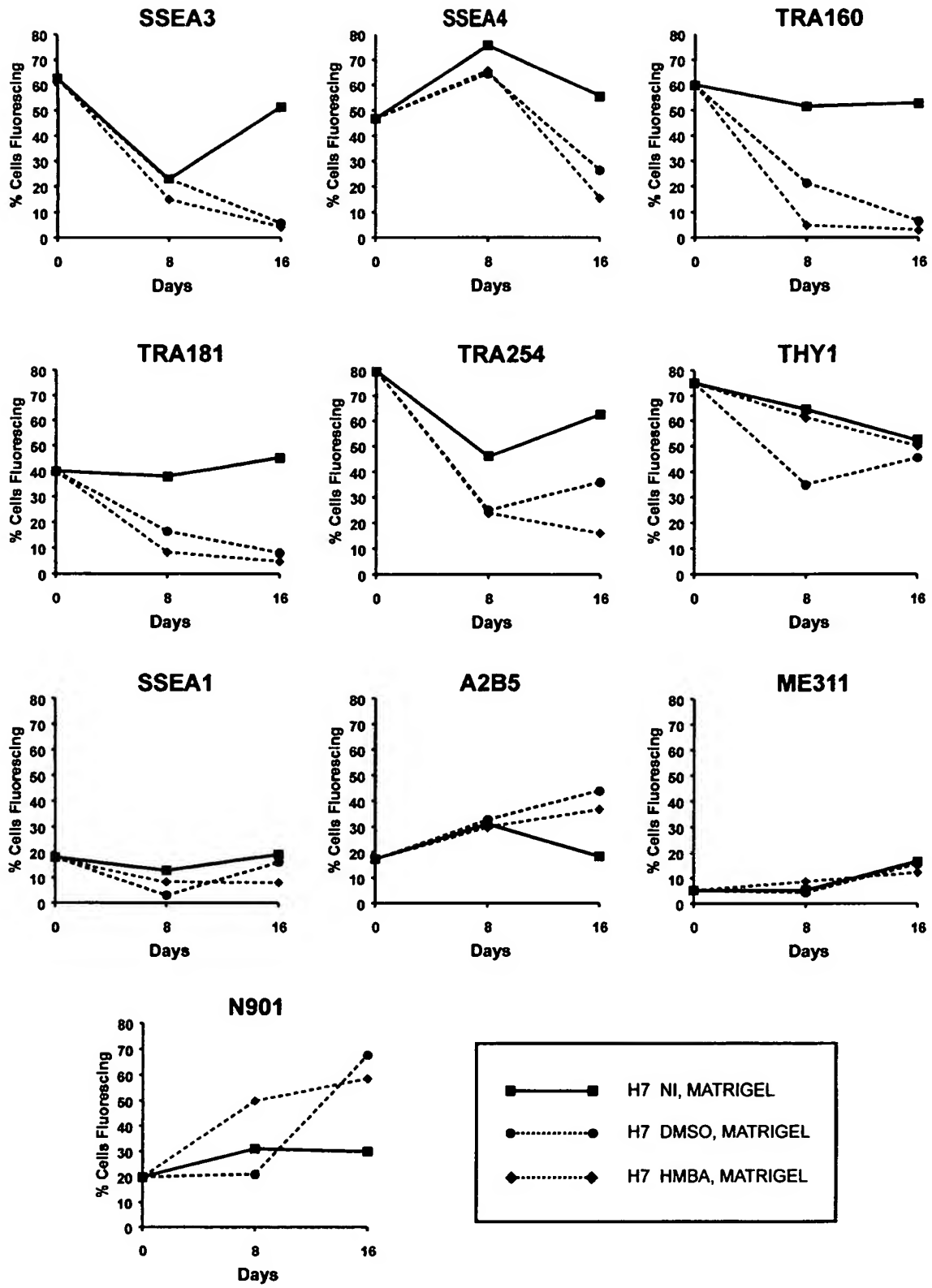

Fig. 5 Surface antigen expression by hES. H7 cells were cultured on Matrigel in the presence, or absence, of 3 mM HMBA or 1% (v/v) DMSO.

SSEA4(+), differing from that of murine EC cells, which are typically SSEA1(+) but SSEA3(–) and SSEA4(–) (Andrews, 1998). A further difference is the definite but low level expression of the class 1 MHC antigens by human EC cells, but not by mouse EC cells (Andrews et al. 1981, 1982, 1984b, 1996). Because of these differences from mouse EC and ES cells it was unclear whether human ES cells derived from human embryos would prove distinct from human EC cells from teratocarcinomas, or whether human ES and EC cells would resemble one another, so that the differences from the mouse would represent differences between the embryos of the two species.

Recent reports, as well as the present study, confirm the similarity of human EC and ES cells, and the existence of differences between comparable cells of humans and mice. Thus, human EC and ES cells are both characterized by their expression of SSEA3, SSEA4, TRA-1-60, TRA-1-81 antigens, as well as the liver/bone/kidney isozyme of alkaline phosphatase (detected by TRA-2-54) and human Thy1 antigen. We have now also demonstrated that these antigens are down-regulated during the differentiation of human ES cells, and that several antigens induced during the differentiation of human EC cells are also induced during the differentiation of ES cells. On the other hand, there are some differences. Whereas several gangliosides are induced during differentiation of human ES cells as detected by antibodies VIN-15-56 and VIN-2PB-22 (GD3 and GD2), other ganglioside antigens that are strongly expressed during NTERA2 human EC differentiation, GT3 detecting A2B5 and 9-O-acetyl-GD3 detecting ME311, were only induced to relatively low levels. Most likely, this reflects rather more heterogeneity within the differentiating human ES cultures. The marked ganglioside expression is most likely particularly associated with neuroectodermal differentiation which occurs in both hES and NTERA2 cultures. However, whereas the hES cells clearly generate muscle and mesodermal derivatives, there is no evidence that NTERA2 is capable of mesodermal differentiation (Gokhale et al. 2000)

The expression of HLA by the human ES cells and its tendency for slight down-regulation upon differentiation is another feature that they share in common with human EC cells. In both cases HLA is strongly induced by IFNγ while amongst the differentiated cultures, the induction of HLA by IFNγ is considerably greater than in the undifferentiated cells. Indeed, IFNγ can also induce the expression of MHC antigens in some mouse EC cells, even though expression is generally not detectable in the absence of this inducer. In both murine and human EC cells it has been shown that these cells only exhibit a partial response to IFNγ without a fully fledged antiviral response (Andrews et al. 1987).

Surface antigen markers provide invaluable tools for monitoring the progress of differentiation and isolate subsets of cells to explore their functions and capacity for further differentiation. Many of the antigens we have studied are associated with carbohydrate epitopes, some linked with glycolipids (e.g. the SSEA series), some with glycoproteins (TRA-1-60 and TRA-1-81). Others are associated with protein epitopes, notably TRA-2-54 (alkaline phosphatase), Thy1 and N901 (NCAM, CD56). The function of the carbohydrate antigens is particularly uncertain in the light of differences in expression between corresponding human and mouse cells. Despite the lack of a clear function for the carbohydrate antigens, a considerable number of genes are devoted to encoding glycosyltransferases, and the expression of these antigens during embryogenesis and cell differentiation is very carefully controlled. It has been suggested that the core structures of these carbohydrates, rather than the terminal structures commonly recognized as epitopes by the various antibodies used in these studies, are the key molecules to affect cell behaviour (Fenderson et al. 1987). Although there are differences in expression of SSEA1, SSEA3 and SSEA4 between mouse and human, globoseries structures are a common feature of these cells in both species: although murine EC cells do not express SSEA3 and SSEA4 they do strongly express the Forsman antigen, another globoseries structure that is absent from human cells (Willison et al. 1982). Some carbohydrate antigens, however, have been shown to have a function. For example, the Lewis-X (Le^x) structure recognized as SSEA1 (Gooi et al. 1981) may be involved in compaction at the morula stage of mouse embryo development (Bird & Kimber, 1984; Fenderson et al. 1984). A role for the carbohydrate structures has also been postulated in axon guidance in the developing nervous system (Dodd & Jessell, 1986), and we previously proposed that the ability of cytomegalovirus to induce inappropriate expression of SSEA1 might provide a partial explanation for its ability to disrupt neural development in infected fetuses (Andrews et al. 1989).

Our present study highlights both similarities and differences between EC and ES cells, from humans, and between these cell types in humans and mice. EC cells

are caricatures of ES cells, adapted for tumour growth, and indeed many human EC cells do not differentiate significantly, presumably reflecting the selective advantage of accumulated mutations that interfere with differentiation (Andrews & Goodfellow, 1980; Duran et al. 2001). Nevertheless, the similarities of differentiation demonstrated by surface antigen changes between human EC cells that do differentiate, like NTERA-2, and hES cells is notable. The patterns induced by RA and HMBA are comparable, even if DMSO does not seem to be an effective inducer of the EC cells. It is also notable that neuroectoderm differentiation is a common feature of EC and hES differentiation, even though the hES cells evidently generate a greater diversity of cell types. Therefore, while it is clear that studies of hES cells can provide insights into the process of development in a way pertinent to human embryogenesis, human EC cells can in some circumstances provide convenient surrogates, lessons from which can be applied to understanding ES cell biology.

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Brachyury Is Expressed by Human Teratocarcinoma Cells in the Absence of Mesodermal Differentiation[1]

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Abstract

Reverse transcription-PCR and Northern and Western blot analyses indicate that mRNA and protein encoded by the *Brachyury* gene are expressed by the pluripotent human embryonal carcinoma cell line NTERA2 and are only modestly down-regulated during retinoic acid-induced differentiation. This differentiation occurs along a neural lineage, with no obvious evidence of the formation of mesodermal derivatives. Several other human embryonal carcinoma cell lines that do not differentiate, a yolk sac carcinoma cell line and two choriocarcinoma cell lines, also express readily detectable levels of *Brachyury* mRNA and protein. Thus, in human teratocarcinomas, *Brachyury* expression is not necessarily an indicator of commitment to mesodermal differentiation.

Introduction

Teratocarcinomas are a subset of GCTs,[3] the most common type of cancer occurring in young men. They contain a disorganized array of embryonic and extraembryonic tissues and provide a paradigm for the notion that the development and progression of cancer involve aberrations in the same mechanisms that regulate cell differentiation during embryogenesis (1). Typically, teratocarcinomas contain highly malignant stem cells, EC cells, that differentiate to form multiple cell lineages leading to a range of terminally differentiated cells, often with the loss of malignancy, in a caricature of embryogenesis (2). In the laboratory mouse, it has long been known that EC cells closely resemble ES cells of the early embryo, and they have been widely used as tools for investigating aspects of embryonic cell differentiation (3, 4). Likewise, recent studies have confirmed the similarity of human tumor-derived EC cells to human embryo-derived ES cells (5, 6). However, it is also evident that human EC and ES cells differ from their murine counterparts. For example, human EC and ES cells characteristically express the globoseries glycolipid antigens SSEA3 and SSEA4 but not the lactoseries glycolipid antigen SSEA1 (6, 7), whereas murine ES and EC cells are typically SSEA3(−) and SSEA4(−), but SSEA1(+) (8–11). Furthermore, human EC and ES cells commonly seem able to differentiate into trophectoderm, a pathway that is, apparently, usually closed to murine EC and ES cells (6, 7).

Many human EC cell lines have apparently lost the capacity for differentiation, perhaps because genetic variants that cause the loss of pluripotency have a selective advantage for tumor growth, because differentiation often appears to result in loss of a malignant phenotype. However, NTERA2 is one human EC cell line that does differentiate extensively and irreversibly in response to retinoic acid to yield a variety of terminally differentiated cells including neurons (12). This differentiation is marked by loss of characteristic EC cell surface antigens and acquisition of new differentiation-specific antigens (13) and by the induction of various developmentally regulated genes, such as those of the *HOX* cluster, which are activated in a retinoic acid dosage-dependent manner (14, 15). Another developmental gene activated during NTERA2 differentiation is *Wnt13* (but not *Wnt1*), in contrast to the differentiation of some murine EC cells (16).

Although the most noticeable cells in differentiated NTERA2 cultures are postmitotic neurons that express a variety of distinctive neural features (12, 17–19), only a small fraction (typically <10%) of the differentiated cells adopt this phenotype. Indeed, many of the differentiated cells derived from NTERA2 EC cells appear to be nonneural, and questions remain as to their identity. One possibility is that some of these nonneural, differentiated NTERA2 cells represent a mesodermal lineage, although no evidence of skeletal muscle differentiation has been forthcoming (12). Accordingly, we sought to test this hypothesis by examining the expression of *Brachyury* during NTERA2 differentiation.

Brachyury (or *T*) was first identified in the laboratory mouse as a dominant short tail mutant that is also a recessive lethal; homozygous *T/T* embryos die in mid-gestation due to a failure of posterior mesoderm (20, 21). Following cloning of the murine *Brachyury* gene (22) and its homologues in other species (23–29), *Brachyury* has generally proved a valuable marker for recognition of mesodermal differentiation (30). For example, apart from expression in embryos themselves, *Brachyury* has been reported to be activated during the differentiation of certain murine EC and ES cell lines differentiating along mesodermal lineages *in vitro* (31–33).

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[3] The abbreviations used are: GCT, germ cell tumor; RT-PCR, reverse transcription-PCR; EC, embryonal carcinoma; ES, embryonic stem; MOPS, 4-morpholinepropanesulfonic acid.

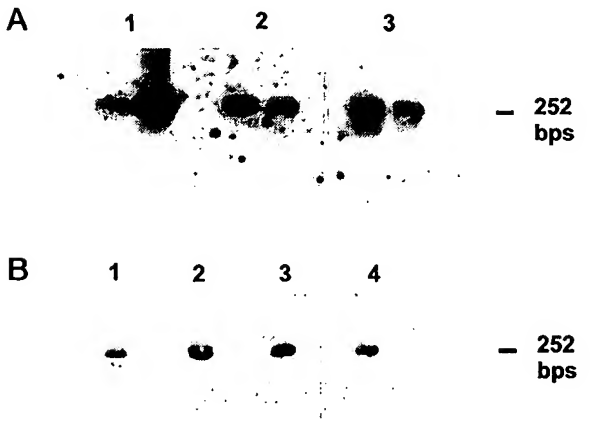

Fig. 1. PCR analysis of *Brachyury* expression in differentiating NTERA2 cells induced with retinoic acid. *A*, RT-PCR from duplicate reactions with RNA isolated from NTERA2 EC cells (*Lane 1*) and cells differentiating in response to retinoic acid for 4 and 14 days (*Lanes 2* and *3*). *B*, *Brachyury* representation in cDNA libraries (34) prepared from NTERA2 EC cells (*Lanes 1*), ME311(+) retinoic acid-induced NTERA2 cells (nonneural; *Lane 2*), and purified NTERA2 derived neurons (*Lane 3*). The PCR products were detected by Southern blotting.

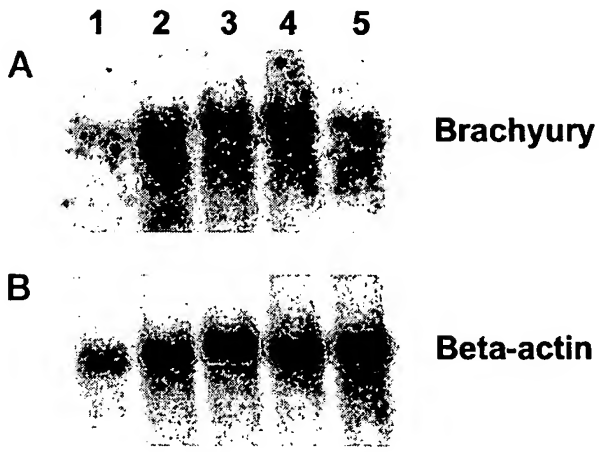

Fig. 2. A, Northern blot analysis of *Brachyury* mRNA expression in NTERA2-derived cells. *Lane 1*, purified neurons; *Lane 2*, cells treated for 12 days with retinoic acid; *Lane 3*, cells treated for 8 days with retinoic acid; *Lane 4*, cells treated with a 4-day retinoic acid treatment; *Lane 5*, EC cells. *B*, β-actin control probe.

The human homologue of the mouse *Brachyury* has been cloned, and its expression was detected by RT-PCR in the notochord remnant, the nucleus pulposus, of human abortuses at 14–15 weeks gestation, but not in the fetal intestine or muscle, or in 14-week spinal cord (29). Both the gene and its predicted protein show strong homology to the mouse *Brachyury*. It was therefore with some surprise that we discovered that *Brachyury* is expressed generally by undifferentiated human EC cells as well as by the differentiating derivatives of NTERA2 EC cells and by yolk sac carcinoma and choriocarcinoma cells.

Results

***Brachyury* Is Detectable in the Pluripotent Human EC Cell Line NTERA2.** To determine whether *Brachyury* is expressed and developmentally regulated in differentiating cultures of NTERA2 EC cells, RNA from the undifferentiated EC cells and from the cells induced to differentiate with 10^{-5} M retinoic acid was initially analyzed by RT-PCR. A product of the expected size of 252 bp was obtained in each case (Fig. 1*A*). The RT-PCR product was confirmed to correspond to *Brachyury* by Southern blotting using a specific probe generated from a plasmid containing the first exon of human *Brachyury*, provided by Dr. D. Stott (University of Warwick, Coventry, United Kingdom). Finally, the PCR product was cloned and shown to have a sequence identical to that of corresponding segment of human *Brachyury* exon 1.

When cultures of NTERA2 cells are induced to differentiate with retinoic acid, they become heterogeneous, and, in addition to neurons, a number of subsets of nonneuronal cells expressing different surface antigens can be seen (13). We previously prepared representative cDNA libraries from undifferentiated NTERA2 EC cells, NTERA2-derived neurons, and a nonneuronal differentiated NTERA2 cell type defined by expression of the cell surface antigen ME311 (34). We now found that *Brachyury* was readily detectable in all three cDNA libraries, including that derived from purified neurons (Fig. 1*B*). Thus, these results provided no evidence of *Brachyury* restriction during NTERA2 differentiation.

To confirm the PCR results in a more quantitative manner, Northern blot analysis was carried out on mRNA isolated from differentiated NTERA2 cells. *Brachyury* mRNA was clearly detected in the untreated EC cells and, with little change in levels, on retinoic acid induction (Fig. 2). The *Brachyury* message also appeared to persist in the purified neuronal preparation (Fig. 2, *Lane 1*), although at a very low level compared with the other stages of NTERA2 differentiation.

***Brachyury* Is Expressed in Several Human Cell Lines Derived from GCTs and Gestational Choriocarcinomas.** In many ways, NTERA2 cells are generally typical of other human EC-derived cell lines, particularly with respect to the characteristic cell surface antigens they express (35). On the other hand, they are among the few established human EC cell lines that show a marked ability to differentiate. The fact that NTERA2 EC cells express *Brachyury* might therefore be a consequence of leaky expression of developmentally important genes, which could in turn reflect their greater capacity for differentiation, rather than a general property of human EC cells. Alternatively, cultures of NTERA2 do show some heterogeneity, hence the presence of cells that have started to differentiate cannot be ruled out. Therefore, we assessed the expression of *Brachyury* in other human EC cells that do not differentiate, as well as in several other non-EC cell lines derived from testicular GCTs. The gestational choriocarcinoma-derived cell lines JAR and BeWo were included to represent the trophoblastic elements commonly found in these cancers.

RT-PCR indicated general expression of *Brachyury* in all of the other human EC and other GCT-related cell lines tested (data not shown), and Northern blot analysis revealed a mRNA transcript of approximately 2 kb corresponding to

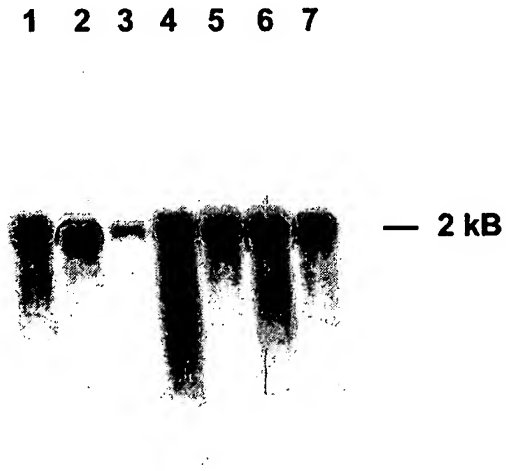

Fig. 3. mRNA (5 μg/lane) was electrophoresed from a variety of cell lines of germ cell origin and subjected to Northern analysis. *Lane 1*, BeWo; *Lane 2*, TERA1; *Lane 3*, 577 MF; *Lane 4*, 2102Ep; *Lane 5*, 1156QE; *Lane 6*, 833KE; *Lane 7*, JAR.

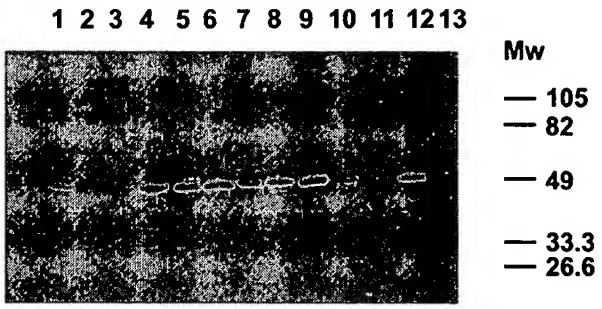

Fig. 4. Western blot analysis of BRACHYURY protein expression in various human GCT- and choriocarcinoma-derived cell lines: NTERA2 EC cells (*Lane 1*); NTERA2 cells differentiated in retinoic acid for 8 days (*Lane 2*) and 13 days (*Lane 3*); 2102Ep EC cell clones cl.4D3 (*Lane 4*) and cl.2A6 (*Lane 5*); 833KE (*Lane 6*); TERA1 (*Lane 7*); 1156QE (*Lane 8*); 1777N RpMet (*Lane 9*); 1411H (*Lane 10*); BeWo (*Lane 11*); JAR (*Lane 12*); and 577 MF (*Lane 13*).

human *Brachyury* in each case (Fig. 3). Thus, *Brachyury* expression seems to be a typical feature of a wide range of these GCT-related cell lines, including several EC cells that do not differentiate significantly.

The unexpectedly wide expression of human *Brachyury* mRNA might be less striking if it were not translated into protein in some cells. We therefore addressed this possibility by Western blot analysis. Although a specific antibody to human BRACHYURY was not available, the high degree of amino acid homology between the mouse and human *Brachyury* sequences suggested that an antimouse BRACHYURY antibody would cross-react sufficiently with the human protein. Using a polyclonal antibody to the NH_2-terminal of mouse BRACHYURY, a band of the expected size for the BRACHURY protein (M_r 50,000–55,000), was detected in lysates of all human EC cells and other GCT-related cell lines correlating with their expression of *Brachyury* mRNA (Fig. 4). In fact, the level of BRACHYURY protein expression in the undifferentiated NTERA2 EC cells (Fig. 4, *Lane 1*) was significantly lower than that seen in the other EC cell lines that do not differentiate in response to retinoic acid (36), 2102Ep (clones 2A6 and 4D3), 833KE, 1156QE, 1777NRPmet, and TERA1 (*Lanes 4–9*). Furthermore, differentiation of NTERA2 cells did result in substantial down-regulation of BRACHYURY protein levels (*Lanes 2* and *3*).

Discussion

PCR, Northern analysis, and Western blotting all indicate that human EC cells express the *Brachyury* gene at significant levels and that the mRNA is translated into protein. Northern analysis of *Brachyury* mRNA in NTERA2 EC cells revealed a single transcript of the anticipated size, ~2 kb, indicating no unusual splice variants in these cells, and the protein detected by Western blotting was also of the anticipated size. A modest down-regulation, particularly of BRACHYURY protein levels, was seen during retinoic acid-induced differentiation of the pluripotent NTERA2 EC cells, although no segregation between neural and nonneural lineages was apparent from analysis of a set of NTERA2 cDNA libraries. Furthermore, expression was evident in cell lines corresponding to the extraembryonic tissues of the yolk sac and trophectoderm.

Several additional genes containing a consensus sequence, the T-box, in common with *Brachyury* have been reported, notably the T-box genes, *Tbx1–5* (37). However, it is very unlikely that any of these were detected in the present experiments. The RT-PCR reactions and the Northern analyses were tested by hybridization with a probe derived from the first exon of human *Brachyury*. Analysis using a BLAST search algorithm[4] indicated that the probe would only detect the published human *Brachyury* sequence and not sequences derived from other related human T-box genes. Furthermore, sequencing of RT-PCR-derived clones indicated that the amplified DNA did indeed correspond to human *Brachyury*.

With the exception of NTERA2, the EC cell lines studied show little or no evidence of an ability to differentiate (7, 8, 36, 38). Previously, we had failed to find expression of desmin in retinoic acid-induced NTERA2 cells (12), whereas we have also failed to find evidence of MyoD or cardiac actin,[5] again implying the absence of muscle differentiation Thus, there is no evidence for a propensity for mesodermal differentiation by the human EC cells studied, despite their expression of *Brachyury*.

The expression of *Brachyury* by undifferentiated human EC cells may appear somewhat surprising. During the embryogenesis of several species, *Brachyury* expression has been specifically associated with the appearance of mesodermal precursor cells (30). For example, in the mouse, *Brachyury* is expressed in the cells adjacent to the primitive streak from the onset of gastrulation and in the primitive streak cells induced by the endoderm to form the mesoderm,

[4] www.ncbi.nlm.nih.gov.
[5] Unpublished results.

and it continues to be expressed as these cells differentiate (39). In *Xenopus* embryos, *Brachyury* is evidently sufficient to induce mesoderm (40). On the other hand, studies of chimeric *T/T* ↔ +/+ mouse embryos suggest that *Brachyury* is not required for the formation of mesoderm in mammals but rather plays a role in migration of these cells from the primitive streak (41). Consistent with this view is the observation that although *Brachyury* is substantially up-regulated when P19 murine EC cells are induced to differentiate into mesodermal derivatives, its overexpression alone is not sufficient to cause the formation of mesoderm cells (31). Indeed, although *Brachyury* marks embryonic cells that become mesoderm, its expression occurs at such an early stage of their commitment that they are still competent to form other cell types (42). Thus, *Brachyury* expression may not necessarily be a marker for commitment, but rather one of competence for mesodermal differentiation.

Recent studies of mouse ES cells in which E-cadherin expression was disrupted suggest a corresponding induction of *Brachyury* expression (43). Indeed, we have found that NTERA2 EC cells express particularly low levels of E-cadherin (44), and we wondered whether this might contribute to the expression of *Brachyury* in these cells. However, the other human EC cell lines that we find express *Brachyury* also express substantially higher levels of E-cadherin (44). Therefore, it seems unlikely that low E-cadherin expression can explain the general expression of *Brachyury* by human EC cells.

The significance of readily detectable levels of *Brachyury* in the range of human embryonic tumors studied here is unclear, but this study serves to indicate further differences between these human tumors and their murine counterparts. One possibility is that the results reflect the tumor derivation of EC cells and are not necessarily indicative of the phenotype of human embryo-derived ES cells. On the other hand, at least with respect to expression of characteristic surface antigens, the phenotypes of human EC and ES cells are reported to be similar (5). Low levels of *Brachyury* have been reported in some murine EC cell lines (31), but we are not aware of evidence of expression in the extraembryonic cells to which choriocarcinoma and yolk sac carcinomas correspond. Given that *Brachyury* expression in murine embryonic cells is associated with competence for mesoderm differentiation, its presence in nullipotent human EC cells might indicate a similar competence, although the corollary of their nullipotency would then be an active repression of differentiation in such cells. Such a repression would clearly have a selective advantage for faster, more aggressive tumor growth.

Materials and Methods

Cell Culture. NTERA2 clone D1 (NTERA2) human EC cells were maintained as undifferentiated stock cultures in DMEM supplemented with 10% FCS as described previously (45). To induce differentiation, these cells were harvested using trypsin:EDTA (0.25% trypsin; 1 mM EDTA) and seeded at 10^6 cells/75-cm^2 tissue culture flask in medium containing 10^{-5} M all-*trans* retinoic acid (Eastman Kodak) (12). Neurons were purified from retinoic acid-induced differentiated NTERA2 cultures after 3–4 weeks using the technique described by Pleasure *et al.* (19).

Several other cell lines derived from human testicular GCTs were also studied. These included the EC cell lines 1156QE, 2102Ep, TERA1, and 833KE (8, 38, 46, 47); the yolk sac carcinoma cell line 1411H (48); and the malignant teratoma cell line 577 MF (38). In addition, we also analyzed the gestational choriocarcinoma cell lines JAR (49) and BeWo (50).

PCR Analysis. Polyadenylated RNA was reverse-transcribed using 300 units of Moloney murine leukemia virus reverse transcriptase (Promega) for 2 h at 37°C in the presence of poly$(dT)_{18}$/random primer, 200 μM deoxynucleotide triphosphate in 50 μl of reaction buffer [final concentration, 50 mM Tris-HCl (pH 8.5), 40 mM KCl, 10 mM DTT, 7 mM $MgCl_2$, and 0.1 mg ml^{-1} BSA]. For PCR, RT cDNA (50 ng) derived in this way or pooled cDNA obtained from cDNA libraries was incubated with 20 pmol of primers (size, 20–30 mer), 200 μM each deoxynucleotide triphosphate, PCR reaction buffer, 2.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, and distilled H_2O to 30 μl.

PCR primers corresponding to exon 1 of human *Brachyury* (GenBank accession number NM003181; Ref. 29) were as follows: (*a*) *Brachyury*, forward primer 5′-TAAGGTGGATCTTCAGGTAGC-3′ (bp 127–146 of GenBank accession number NM 003181); and (*b*) *Brachyury*, reverse primer 5′-CATCTCATTGGTGAGCTCCCT-3′ (358–377 bp).

The following PCR cycle was used: (*a*) 93°C, 3 min (1 cycle); (*b*) 94°C, annealing temperature (58°C), 1.5 min, 72°C, 1.5 min (35 cycles); and (*c*) 72°C, 5 min (1 cycle).

PCR products were analyzed by electrophoresis on 1% agarose gels containing 1× Tris-acetate-EDTA buffer. Selected DNA bands were excised, purified by ethanol precipitation, and cloned by ligating into precut T/A vectors (Promega) that were used to transform DH5-α *Escherichia coli*. Cloned DNA fragments were sequenced using the Prism fluorescence-labeled chain terminator sequencing kit (Perkin-Elmer) and analyzed by the in-house sequencing service (Krebs Institute, University of Sheffield, Sheffield, United Kingdom).

Southern and Northern Blot Analyses. For Southern blotting, DNA was separated on horizontal 1–1.5% agarose gels and transferred in 0.4 N NaOH to Hybond membranes (Amersham-Pharmacia, Ltd.). ^{32}P-labeled DNA probes were hybridized at 65°C in 5× saline-sodium phosphate-EDTA, 5× Denhardt's solution, 0.5% (w/v) SDS, and 0.5 mg of denatured salmon sperm DNA.

For Northern analysis, 5 μg of mRNA were loaded per lane on a 1% MOPS acid gel (51; 3–5 mm thick) and electrophoresed in 1× MOPS buffer [0.04 M MOPS, 0.01 M sodium acetate, and 1 mM EDTA (pH 7.2)] at 60 V for 3–4 h. RNA was transferred in 10× SSC to a Gene Screen Plus nylon membrane (DuPont). Hybridization was performed at 42°C in 50% (w/v) formamide, 5× saline-sodium phosphate-EDTA, 5× Denhardt's solution, 1% SDS, and 10% dextran sulfate sodium salt (M_r 500,000). Hybridization in Southern and Northern analyses was visualized by a Bio-Rad phosphorimager.

Western Blot Analysis. Monolayers of cells were rinsed three times with ice-cold PBS and incubated with 1 ml of lysis buffer/75-cm^2 flask (1% v/v NP40, 1% w/v sodium deoxycholate, and 0.1 mM phenylmethylsulfonyl fluoride in PBS) for 15 min at 4°C. Cell lysates were passed through a 21-gauge needle to shear the DNA, followed by a freeze/thaw cycle and centrifugation (30 min, 4°C, 15,000 × *g*) to remove insoluble material. Protein concentrations were determined using the Bio-Rad assay. SDS-PAGE was carried out by the method of Laemmli (52) using 16 μg of protein per lane of a 10% polyacrylamide gel. Subsequently, the separated proteins were transferred electrophoretically to a nitrocellulose membrane (pore size, 0.45 μm), which was then washed with PBS and 0.05% Tween (PBS-T) and blocked using 5% milk powder dissolved in PBS-T (60 min, room temperature). The blots were incubated with a purified polyclonal antibody raised against a peptide composed of the first 129 amino acids of mouse BRACHYURY protein (24). After washing, the blots were incubated with horseradish peroxidase-labeled secondary antibody; and antibody was visualized by using the enhanced chemiluminescence technique (Amersham-Pharmacia).

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